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(From the Microbiological Laboratory, Agricultural University, Wageningen.)

THE *B. COLI* TEST IN THE ROUTINE ANALYSIS OF RAW MILK

by

JAN SMIT, B. M. KROL and A. J. VAN WIJK.

(Received June 15, 1939).

The estimation of *B. coli* in milk serves a purpose different from that in the control of drinking water. In fact, whilst purification of our potable waters aims at the elimination of every trace of contamination with faecal material, of which *B. coli* is the main testifyer, the presence of this bacterium in fresh milk is regarded as unavoidable. So the amount of milk, wherein its absence has to be proved, has become the main point of interest. As well as in other countries also in the Netherlands this quantity is open to differences of opinion: whilst several municipal authorities are satisfied when *B. coli* is absent in 0,001 ml., others maintain the view, that a few hygienic measures would enable the farmers to increase this volume up to 0,01 ml. or even to 0,1 ml. Leaving this point out of discussion here, we emphasize two facts:

1) The estimation should be completed in the shortest time possible. The daily control of the milk of numerous farmers, as practiced in all dairy factories, and in most of the Food Control Stations in Holland, is inappropriate when it takes two days or more to fix the result, and a one day test of a slightly lower sensitiveness is to be preferred to a very sensitive estimation taking a longer time to perform.

2) Given the quantitative character of the *B.coli* test, every classification of the coliform bacteria should be avoided: it is of no importance, whether a positive result of the chosen "*B. coli* test", whichever this may be, is due to a "true" *B. coli* or to a representative of the *Aerobacter*- or Intermediate group, as long as we know that pure milk is free of any of them; so every member of the colon group suits the purpose. Consequently only the properties common to the whole group are of value for this test: all of them are facultative anaerobic, gram negative, non chromogenic short rods without spores, fermenting glucose and lactose with the formation of acid and gas. Other properties: indole-, methylred- and citrate test e.t.q. are of minor importance.

The number of *B. coli* tests used in milk analysis is fairly large: the Standard Methods of Milk Analysis of the American Public Health Association, the "Österreichisches Lebensmittelbuch", the excellent monograph of WILSON and co-workers (1), and several other textbooks on milk (2) describe various tests, differing from each other in many respects, but most of them corresponding in the fact, that at least 48 hours are needed to complete them. A short description follows here:

A. Standard Methods A.P.H.A.: A standard test for the colon group is described, using Durham tubes, containing a brilliant green-lactose-peptone-bile-solution. The tubes are provided with different milk dilutions, and are incubated at 37° C. for 2 days. Those tubes containing 10 percent of gas or more are deemed positive, those with less than 10 percent negative or doubtful. From the number of positives (what becomes of the "doubtfuls" is not stated) a "most probable number" of *B. coli* is made up in the usual way. Any conclusions from the numbers found are lacking. The standard test takes 48 hours to complete.

B. WILSON distinguishes between the dilution method and the plate method. Of the former, 3 different modifications are described, and only one of the latter, but in all of them the same nutrient solution is used, namely the Mac Conkey broth, which contains 0,5 % sodiumtaurocholate, 1 % lactose, 2 % peptone, 0,5 % NaCl and 1 % of a 1:100 solution of neutral red. Distilled water is used throughout. If the plate method is used, this solution is solidified with 2 % agar. Of the 3 dilution methods described, one, number III, is specially preconized for a rapid faecal coli count. According to this method one set of the dilutions is incubated at 37° C., another set at 44° C. From the result noted after 48 hours (without taking notice of the amount of gas) the most probable number is made up in the usual way. Positives at 37° C. are transferred into citrate broth, and again incubated at 37° C. during 48 hours. Growth in this medium indicates the presence of members of the *Aerobacter* group. The whole procedure takes at least 3 days.

Using the plate method, two 1 ml. portions of the different dilutions are mixed with the agar described, and one of each is kept at 37° C., the other at 44° C. After two days the red colonies are counted, those on the 44° C. plates being regarded as "true faecal *B. coli*".

WILSON's own judgment of this test is severe: "The objection to this method is that the coliform count tends to be too high, since a

proportion of the organisms developing on the 37° C. plates may not be coliform bacilli at all." (Cf. (1) p. 207).

C. The "Osterreichisches Lebensmittelbuch, XLV Heft (1936)" uses "trypsin broth", *i.e.* a solution of 1 % peptone (Witte) and 0,5—1 % meat extract (Liebig), digested during 24 hours with trypsin at 37° C. Indole formation within 24 hours is said to indicate the presence of *B. coli* whilst other indole producing bacteria like *B. proteus* are "improbable". *Aerobacter* is not shown. For the estimation of both *Escherichia* and *Aerobacter* two media are recommended: *viz.*, 1) the trypaflavine-bromothymol blue-agar after KLIMMER, HAUPT and BORCHERS (3), and 2) the gentian violet-lactose-bile-peptone of KESSLER and SWENARTON (4). The solution is tubed in Durham tubes, and any gas formation in 24 hours is said to indicate coliform organisms.

D. In 1932 the Prussian Government (5) prescribed the use of lactose-bromothymol blue-trypaflavine-agar for the *B. coli* count, by spreading 0,1 ml. of milk on the well dried agar, and counting after 18 hours at 37° C. It was, however, stated by RITTER (6) that this test is not specific enough, and gives a much too high count, especially when the milk is spread on the solid agar, in stead of being mixed with the melted medium.

E. Besides the above mentioned media, those of TONNEY and NOBLE (10) (ferrocyanide-citrate-agar), and the erythrosin-methylene blue-bromo-cresol purple-trypaflavine-lactose-agar (Cf. (2) p. 147) may be cited. All of them aim at the development of *B. coli* in media, which are unsuitable to other milkbacteria, and thus claim the possibility of a direct coli count by exclusion of all others.

F. The method of GERSBACH (7) consists in estimating the dilution in which indole is formed when incubated at 37 ° C. in a peptone solution. Like the above mentioned method with trypsin broth it should therefore be called "indole test", and it is clear, that it will not necessarily correlate with the number of *B. coli* present, because indole-negative coliforms, *Aerobacter* included, are fairly numerous in milk, whilst on the other hand several indole producing bacteria like *B. proteus* may be present, thus increasing the number of indole forming species. Notwithstanding these facts, this method (originally designed for use in water analysis) has proved to give a fairly accurate indication of the number of *B. coli* in milk by a simple and inexpensive procedure, which, it is true, takes 48 hours to perform, although it is found that the result after 24 hours in many cases closely agrees with that after two days. Thus VAN RAALTE (11) stated

that for the Amsterdam milk supply a positive GERSBACH test with 1/100 ml. is normal, where hygienic conditions on the farms are fairly good, an eventual drop to 1/1000 ml. being allowed. Although this method does not claim to be a universal quantitative coli test, it seems to be applicable in every instance, where a certain standard of purity is to be maintained, and where a test of short duration is needed to check the results. It does not make any difference whether the limit aimed at lies at 1/100 ml., or that a more severe standard, say 1/10 ml., may conveniently be prescribed, as long as this standard meets the hygienic conditions prevailing, and may be tolerated under the existing conditions. And whether or not this indole test completely correlates with a more severe coli test seems to be of minor importance. We will see further down, that the results of Gersbach's test generally follow the values found in the different coli tests very closely, and that the differences are in the same direction.

G. The Dutch "Melkbesluit" (Milk act, June 1923) does not contain any prescriptions concerning the estimation of *B. coli* in raw milk. However its absence from 5 ml. of pasteurized milk is demanded, and a coli test is prescribed, named the Ringeling test, using 50 ml. of non-neutralized broth (called "acid broth") for 5 ml. of milk. The mixture is to be kept at 35° C. for 24 hours, and thereafter it is "checked on the presence of colibacteria". No further particulars are given, but it is clear that plating, e.g. on Endo agar, or a similar solid medium, is meant, which extends the duration of the procedure to 48 hours or more.

In conclusion it must be stated, that, with the possible exception of the trypaflavine plates and the gentian violet-bile-broth, the reliability of which is still open to discussion, all tests cited require a longer time to complete than is deemed suitable for routine analysis of large milk supplies.

Nevertheless it seemed worthwhile to investigate, whether shortening the period of incubation to 24 hours would lead to presumptive results, which might be regarded as acceptable. We tentatively began by making a comparision between the Mac Conkey broth (WILSON), the Gersbach test and a liquid medium of the same composition as the well known Endo agar, expecting to combine the gas formation from lactose with the red colouration, produced on the agar by members of the colon group. A similar medium has already been described by STERN (8), with the aim to

differentiate between all members of the group, *B. typhosum* and *paratyphosum* included.

We used a liquid of the following, slightly changed, composition: In 900 ml. tapwater dissolve: 10 g. Liebig's meat extract; 10 g. peptone (Witte); 5 g. sodium chloride. Bring the pH to 7.6 — 7.8 with Na_2CO_3 solution. Boil, filter and sterilise. Then add: 50 ml. of a 20 % sterile lactose solution; 2.5 ml. 10 % alcoholic fuchsin solution; 1.9 g Na_2SO_3, dissolved in a few ml. of moderately hot water. Make up to 1 litre with sterile water. The clear and only slightly coloured liquid is poured into Durham tubes, and subjected to a final sterilisation. Thereby the liquid becomes deeply stained, but after cooling the red colour fades again.

In these tubes *B. coli* and *A. aerogenes* grow and ferment abundantly and within 24 hours stain the liquid to a deep red. Our first series of experiments with milk included the Ringeling test, and all of the presumptive tests were followed by subculturing on Endo plates. One typical colony was checked in glucose and lactose broth and for indole production in peptone broth. Table I gives the result.

TABLE I.
ESTIMATION OF *B.COLI* IN MILK.

Method	Dilution	Confirmed coli test			
	-2 -3 -4 -5	Dilution used	glu-cose	lac-tose	in-dole
1) Mc Conkey, gas . .	+ + + —				
Confirmation on Endo	+ + + —	—3	+	+	—
2) Gersbach	+ — — —	—2	+	+	+
Confirmation on Endo	+ + — —	—3	+	+	—
3) Ringeling, incl. subculturing on Endo	+ + — —	—3	+	+	—
4) Endo broth, colour .	+ + + —				
„ „ gas . .	+ + + —				
Confirmation on Endo	+ + + —	—4	+	+	—

As is seen from Table I this milk sample showed a positive presumptive test with 10^{-4} ml. in the Mc Conkey and Endo broth test, in the Ringeling and Gersbach tests only with 10^{-3} and 10^{-2} ml. respectively. It will be seen, that from the negative -3 dilutions

in the Gersbach test (no indole), the confirmation on Endo agar was positive, but the colony tested thereupon was indole-negative. The same was true for the colonies picked from the other Endo plates, except for that from the -2 Gersbach plate. Table II gives another example.

TABLE II.

ESTIMATION OF *B. COLI* IN MILK.

Method	Dilution	Confirmed coli test			
	-2 -3 -4 -5	Dilution used	glu-cose	lac-tose	in-dole
1) Mc Conkey, gas . .	+ — — —				
Confirmation on Endo	+ — — —	—2	+	+	—
2) Gersbach	— — — —				
Confirmation on Endo	+ — — —	—2	+	+	—
3) Ringeling, incl. subculturing on Endo	+ — — —	—2	+	—	—
4) Endo broth, colour .	+ + — —				
„ „ gas . .	+ + — —				
Confirmation on Endo	+ + — —	—3	+	+	—

The investigation was extended to 30 samples with the result given in Table III.

TABLE III.

RESULT OF THE INVESTIGATION OF 30 MILK SAMPLES.
NUMBERS OF POSITIVES.

Method	Dilution				
	-1	-2	-3	-4	-5
1) Mc Conkey	30	24	16	8	2
2) Gersbach	24	19	11	6	1
3) Ringeling followed by Endo plate . . .	30	25	21	9	3
4) Endo broth, colour .	30	25	19	10	4
„ „ gas . .	30	25	16	8	3

The following conclusions seem warranted:

1) The Mac Conkey test gives constant and good results as far as gas formation is concerned: a positive test can nearly always be confirmed by plating on Endo agar (the case of Table I forms an exception).

2) The Gersbach test proved less sensitive than the other three: the limit of positives generally stands back by one or even two dilutions. It will be shown hereafter, that the confirmation test gives the explanation of this fact.

3) The Ringeling test gives fair results, in agreement with the other tests. The confirmation on Endo is unavoidable in this case, extending the time of completion to 48 hours, and thus decreasing its usefulness.

4) The test with Endo broth gives the best and most uniform results, allowing a fairly accurate insight into the quality of the milk after 24 hours. Gas formation and development of colour nearly always correlate. The few exceptions were stated in the cases, where colour developed without gas (the reverse never occurred), but in those tubes *B. coli* could always be found.

The results of Table III were slightly modified, when all tubes, the negatives included, were plated on Endo agar. Then the number of positives increased, especially in the Gersbach test, showing that many indole-negative coliforms occur in milk. The agreement of the four tests now proves to be very fair (see Table IV).

TABLE IV.

NUMBER OF POSITIVES, AFTER CONFIRMATION ON ENDO PLATES.

Method (Endo plate included)	Dilution				
	-1	-2	-3	-4	-5
1) Mc Conkey	30	25	18	8	3
2) Gersbach	30	25	18	11	2
3) Ringeling	30	25	21	9	3
4) Endo broth	30	25	18	9	3

If, however, a result after 24 hours is demanded, then Table III holds good (the Ringeling test must be dropped), from which

it may be taken, that the Mc Conkey and Endo broth tests are equally suitable, but that the Gersbach test lags behind. In the Endo test we dispose of two nearly always correlating data: development of colour and gas formation, which in our opinion gives it a certain advantage over the Mc Conkey test. It must be added, that, for unknown reasons, development of fluorescence failed in most of the tubes of the latter test.

It can not be denied, that the number of positives in both tests is smaller after 24 hours than after 48 hours, in agreement with WILSON's results. However, both tests are similar in this respect, and it seems to us, that this fact makes no important difference in the judgment of the milk, because in a limited number of cases only this judgment is slightly too favourable.

The Endo broth has the advantage of being considerably less expensive: the price per liter of Mc Conkey broth is about fl. 1.—, against fl. 0.40 for 1 liter of Endo broth.

If glutamic acid was substituted for the Liebig meat extract (9), equally consistant results were obtained. We succeeded in giving this medium a composition, which allowed it to be powdered, and kept indefinitely in a dry state without changeing its quality:

10 g. peptone (Witte or Difco)
10 g. glutamic acid
10 g. lactose
5 g. NaCl
1 g. K_2HPO_4
4 g. Na_2CO_3 (anhydr.)
0.26 g. fuchsin
1.9 g. $Na_2SO_3.7H_2O$

Of this mixture 42.15 g. are made up to 1 liter with distilled water. Sterilization for 15 minutes at 105° C. is allowed, but in case of immediate use, boiling and filtering, followed by tubing, gives satisfactory results.

Finally we tried to investigate, whether the coliform organisms, isolated from negative Gersbach tubes, inoculated with dilutions of milk, which yielded positive results in other media, must be regarded as indole negative *B. coli* or as belonging to other groups. The result was, briefly stated, that most of the strains isolated were *B. coli* (M.R. +, V.P. —, citrate —, indole —), but that a few strains of

A. aerogenes and intermediates were found. (In some cases indole positive strains were grown from indole negative tubes). The reason of this prevalence of the indole negative coli-types in milk is obscure.

In conclusion we are of opinion, that the quantitative estimation of coliform organisms may be performed in a simple way, within 24 hours giving results, which do not materially differ from those obtained in 48 hours or by confirmed tests of any kind. We consider this quick coli-count as a very efficient routine method for the bacteriological grading of raw milk supplies in factories and control stations, where checking the number of *B. coli* is of importance in addition to other grading methods (Breed method, little plate method, methylene blue test), but where the maximum of accuracy is not aimed at.

The investigation of other methods of short duration, cited above, will form the object of further study.

Summary.

It is emphasized, that the estimation of coliform bacteria is important in the routine analysis of raw milk. Various tests recommended in the literature are described, and stress is laid on the fact, that only those which may be completed in 24 hours are of outstanding value. A prescription is given of a satisfactory test using a broth of nearly the same composition as the well known Endo agar, and a comparison is made of the results of this test with 3 other tests in investigating 30 samples of raw milk. It is proved that a considerable number of the coliforms found are true *B coli* which have lost the power of indole formation.

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(From the Laboratories of Hygiene of the University; of the Tropical Hygiene Department of the Colonial Institute, and of the Health Department of the Municipal Medical and Health Service, all at Amsterdam.)

THE RECOGNITION OF PSITTACOSIS

by

H. VERVOORT and A. CHARLOTTE RUYS.

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At the end of November 1937, we succeeded in isolating the virus of psittacosis by the mouse-test from the sputum of a patient, suffering from an atypical pneumonia, first diagnosed as influenza. This finding has been confirmed by a positive complement-fixation reaction, with a sample of the serum from the patient, after her recovery, performed by Prof. BEDSON in London.

The virus has been kept continuously active bij regular transmissions in mice. The case has been described previously (10).

Since then several sputa (11 samples) from persons with more or less suspected symptoms have been tested with negative results. In 7 the complement-fixation test proved to be negative after the recovery of the patient. In 2 no complement-fixation test has been made. One patient was suffering from Bang's disease.

One sputum was from a patient who died afterwards from a pneumonia. At the post mortem (Dr. DRUKKER, Wilhelmina Hospital) the lungs showed patches of bronchopneumonic consolidation. On section the cut surface was deep red; a sanguinolent fluid and air bubbles could be expressed. The liver was enlarged and fatty; the spleen enlarged, the pulp friable.

Portions of the lung and the spleen were ground up, and suspended in peptone broth. Mice were inoculated intraperitoneally with the suspensions. On the 13th day both "lung-mice" became ill, and refused food. One was killed on the 15th, the other one on the 16th day after the inoculation. The first showed no special lesions; the peritoneal cavity contained some sticky fluid; liver, spleen, adrenals, bowels were unchanged. The peritoneal cavity of the second one contained more sticky fluid; liver and spleen were coated with a sheet of fibrin. No other lesions. In smears from the fluid and from the spleen, scarcely virus could be revealed. Both "spleen-mice" remained negative in two transmissions.

The virus, which is now in the ninth transmission, remains of low virulence, with a preference for inflammation of the peritoneum, and the forming of a fibrinous exsudate.

From two other suspect post mortems, suspensions of portions of the lung and other organs were injected into mice, but no virus could be revealed. There had existed but a distant contact with healthy birds; no complement-fixation test has been made.

In connection with some cases of psittacosis two batches of birds have been examined on the presence of psittacosis virus. There had been disease, and many fatal cases amongst those birds. We succeeded in isolating the virus from a dead budgerigar, a number of 4 healthy exotic starlings, and from a sick ricebird. In the last one the virus could be revealed in smears from the peritoneal fluid and from the spleen.

An effort to isolate the virus from a batch of ten birds, kept in our laboratory for other experiments, amongst which there had been two fatal cases, shortly after they had been bought at the market, failed.

It is remarkable that one of our workers, who stayed daily in the same room as the birds, during a slight affection of the throat showed a lightly positive complement-fixation (1/4 ++), which a fortnight afterwards was even a little stronger (1/4 +++). A sputum test in mice did not reveal any virus *).

The pathological changes in mice, inoculated by intraperitoneal injection, are not always described uniformly. Whilst German writers (Fortner (7), Gerlach (8), Pfaffenberg (12)) speak of a "typical" post mortem, with an enlargement of the spleen and liver, a swelling of the duodenum and a mucofibrinous peritonitic exsudate, Rivers Berry and Sprunt (13) lay the stress on an affection of the liver, with necrotic spots. Burnet (3) in Australia mentions a great tendency to produce peritonitic, rather than other lesions, in mice, inoculated with the virus, isolated from apparently healthy wild birds.

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*) When this paper was already under the press, the presence of the *virus of psittacosis* was demonstrated in a passage from the primary mouse, inoculated with a suspension of the *sputum* from this worker. So there really has been an infection of psittacosis in a very light degree, apparently due to virus from the batch of birds. As the laboratory experiments did not yet come to an end, the observations will be recorded in a following paper.

As far as we have observed, the general pattern is very irregular, and seems to depend mainly on the virulence of the strain, whilst at the same time individual resistence of every mouse is of influence. Moreover, an infection with pneumococcus or an other microbe or virus may give analogous pathological changes.

Our first strain "Ruys" isolated from the sputum from a patient is of medium virulence. It kills a mouse in 4 or 5 days: the "typical" pattern is seen in about 10—20 % of the autopsies; there is very often a cellular peritonitic exsudate, whilst liver and spleen are coated with sheets of white fibrino-purulent lymphe, which can be peeled off, leaving the surface of the organ without lesions.

The other four strains are of much lower virulence, and give many failures. An excess of sticky fluid, containing flakes of fibrin is very often the only anomaly to be seen, whilst the mice are but slightly ill during several weeks.

For examination of the smear for the presence of virus, the best films are obtained with the GIEMSA or the CASTANEDA stain.

In films fixed with metylalcohol, and stained with GIEMSA, there is often an inconvenient deposit of amorphe corpuscles, whilst treating the film previously with distilled water, to dissolve the particles, has a bad influence on the staining afterwards.

The CASTANEDA staining has nearly as many modifications as there are workers. The original prescription (4) is a buffered 5 % formalin solution, with 3 drops Loeffler's alkaline methylene blue solution on 20 ccm. Fixing and staining at the same time during 2 minutes, washing, counterstaining with aqueous safranin during 1 or 2 seconds, washing. The virus is stained blue, the background orange-red. RIVERS (13) uses a stain which contains 5 % Loeffler's solution. The textbook of DOERR and HALLAUER (5) gives an insufficient illustration of the normal CASTANEDA effect, whilst that of DA ROCHA LIMA, REISS and SILBERSCHMIDT (14) gives a different prescription under the flag of CASTANEDA.

We use with success a more neutral methylene blue solution, mentioned by BEDSON (2) as "Borrel blue", and described under the name of "Bleu Borrel" in the encyclopedia of EHRLICH (6): a concentrated solution of methylene blue medicinale (HOECHST)*) in distilled water is treated for some days with a washed deposit of silveroxide, prepared by adding a few drops of normal sodium-

*) now called Aretit.

hydroxide solution to a 10 % solution of silver nitrate. After fixing the film in a 5 % formalin solution, we stain for 2 minutes with the concentrated "Bleu Borrel". After washing we use a concentrated safranin solution (GRÜBLER) as counterstain, for 5 to 10 seconds; then we wash and dry. The same fluid can be used during a long time. In a well stained film the virus is lightly blue, the nuclei orange-red till brown, the cytoplasm orange-red. The fresher the film, the better the result.

With other recommanded virus stains, as Victoria blue (HERZBERG (9)) or isamine blue (NICOLAU (11)) it is not easy to reveal the virus in routine-slides, because there is no contrast.

During our experiments to reveal the virus we formed the opinion, that either our mice are not very susceptible for the virus, or that the virus in the carriers is so weak, that it does not get hold in the mice. At all events, one must not expect, to see "typical" patterns of lesions by intraperitoneal inoculation into mice, but one has to search for the virus, and to consider the appearence of the cells in the peritonitic exsudate. The finding of heavy vacuolised cells, even without virus, is suspect.

The virus can be found in monocytes, as small elementary bodies or in clusters within the vacuolised cytoplasm, or it may lay between the cells. Sometimes one finds apparently normal endothelial cells filled up with virus-clusters; also the polynuclear leucocytes may contain the virus.

Many strains of mice, from different sources, are infected with toxoplasm. This infection is the cause of an enlargment of the spleen, and often also of the liver. There may appear a peritonitic exsudate, which is not so sticky as in psittacosis, and apparently the lungs are in a certain way affected. The toxoplasm also is stained blue, but its aspect is very distinct from the psittacosis virus.

There is an other observation, worth to be mentioned. After fixation o *fresh* smears in formalin, the plasm of some polynuclear leucocytes may be scattered in grains, which show an affinity for methylene blue, especially when the staining has been prolongated. It gives the impression of a virus or a micro-organism, but it does not appear after alcohol fixation. It is so typical, that is does not interfere with the diagnose of psittacosis virus. It appears often (not always) in normal not inoculated, as well as in inoculated mice, and has nothing to do with an ep-erythrozoon infection; after the extirpation of the spleen, the ep-erythrozoon appears in the periferic circulation, but the granulation of the leucocytes remains the same.

The investigation of a case, suspected of psittacosis, by mouse inoculation is not easy, and often fruitless.

As blood commonly is only infectious during the first days of the disease, sputum is the material of choice. A pneumococcus infection may interfere before the virus has developed sufficiently. Very often it is impossible to get a sample of sputum, as this is very scarce in psittacosis. The complement-fixation reaction introduced by BEDSON in London, is of great service for the recognition of the case. Following the prescription, given by BEDSON (1), we succeeded in preparing an antigen from the spleen of mice, inoculated with our first strain. A suspension of mouse spleen, rich in virus is made in M/50 phosphate buffer pH = 7.6; 5 ccm of diluent per spleen is used. *) This suspension is allowed to sediment in the refrigerator for 24 hours, or lightly centrifuged, to remove coarser tissue fragments, and the partially clarified supernatant is then centrifuged for 1.5 hours at 3000 r.p.m. The supernatant fluid is pipetted off, and the deposit is resuspended to the original volume in phosphate buffer. *) Any coarse fragments are removed by light centrifugation, and the suspension is steamed for 30 minutes. Before using, the sediment in the antigen has to be resuspended by shaking. As a control serves a suspension of normal spleen, prepared in the same way. With this antigen it has been possible to examine the serum of several formerly suspected cases.

The complement-fixation test was performed as a micro-method with a WRIGHT's pipet. The antigen consisted of the mouse spleen suspension mixed with the same quantity of saline. The sera under examination were inactivated, and used in dilutions ranging from 1/4 to 1/128.

The minimal hemolytic dose of complement in the presence of antigen is determined in a preliminary test, and an excess of this dose, often the double was used in these experiments. In the beginning when we merely used one minimal hemolytic dose of complement we often found dubious reactions. Perhaps the excess of complement will hide some of the weaker reactions but as long as our knowledge on this subject is scarce we think it wiser to consider only the strongly positive reactions.

In the test we mix one part of antigen, with one part of the serum and one part of the complement-dilution. After one hours fixation in the incubator at 37° C. two parts of sensitized bloodcells are added. After another hours incubation at 37° C. the reading is taken.

In each test controls are introduced with a normal mouse

*) we used buffered saline.

spleen suspension, and also with known positive and negative sera. Prof. BEDSON was so kind to send us some of his antigen for comparison. In many tests we worked with three antigens. Table II shows, that the degree of variation is very small. Prof. BEDSON also tested some of the sera from our patients. The results were always identical. In all our patients the Wassermann reaction was negative.

In March and April 1939 we received the sera from several persons, who had been ill a few months previously with symptoms resembling influenza or pneumonia. They all had been in contact with a batch of birds (canaries, finches, budgerigars and others), which came from a nursery, and went from one family to the other. Among the birds many died from an unknown cause. The epidemic was not traced but several months after its onset, when all the patients had recovered. So it was too late to examine their sputum for the presence of virus, but several blood specimens could be obtained for the complement-fixation test. In Table I the result of this investigation, and the course of the epidemic is related. (The epidemiological investigations were made by A. L. NOORDAM).

During the time the batch of birds stayed with family D. many of them died, and several new ones were supplied. So on March 13th another budgerigar was put in the cage, where is was found dead the next morning. The psittacosis virus could be demonstrated in its spleen by inoculation into mice. The last bird came from family v. D., who had another budgerigar and two ricebirds. One died, the other fell ill in April, and at the autopsy the virus could be revealed directly, and by inoculation into mice. In the other budgerigar no virus could be demonstrated.

In family v. D. the housewife fell ill on March 22th with symptoms of a moderate severe influenza. She called no physician, and had recovered after a week. We could examine her blood 12 days after the onset of the disease and found the complement-fixation test strongly positive (1/64 ++++).

Besides this little epidemic two separate cases of psittacosis were observed last year. One patient suffered from an atypical broncho-pneumonia and died. In his sputum no virus could be demonstrated but it was isolated from a portion of the lungs obtained by autopsy. We were not able to trace the source of this infection.

In the other patient, who possessed a large batch of exotic

TABLE I.

COURSE OF A SMALL EPIDEMIC OF PSITTACOSIS.

Time during which the birds stayed in the family		number of persons in family	number of persons ill	case	date onset of disease	complement-fixation test date	complement-fixation test serum dilution	remarks on patients	remarks on birds
Z.	19 Oct.-15 Nov.	5 (+1)	1(+1)	Z ♀	6 Nov.	7 Febr.	1/16 +++	severely ill	many died
				S ♂	3 Nov.	7 Febr.	1/16 +++	pneumonia (neighbour who handled birds)	
L.	15 Nov.-30 Nov.	4	2	L ♂	25 Nov.	7 March	1/2 —	light illness	many died
				L ♀	25 Nov.	16 March	1/64 +++	severe illness	
v. S.	16 Nov.-Dec.	4	2	v. S. ♀	28 Nov.	24 March	1/16 ++	severe illness	the 4 birds in this family all died in Dec.
				v. S. ♀	28 Nov.	*		severe illness	
F.	30 Nov.-March	2	0	F		17 March	1/2 —		the 2 budgerigars in this family remained healthy, no virus was found.
S.	2-3 Dec.	6	1	S ♀ !	20 Dec.	14 Apr.	1/16 ++++	severely ill	from the whole batch 5 died in one day.
D.	3 Dec.-16 March, when the birds were killed	4	0	D ♀		17 March	1/2 —		virus isolated from mixture of spleen of 4 starlings; in 16 other birds no virus demonstrated.

* no blood obtained.

birds, the disease was diagnosed as a very severe influenza. After an illness of two months he recovered. A few weeks afterwards he met a friend from Stockholm with whom he passed some hours in town but he did not receive him at his home. The friend fell ill two weeks afterwards in Stockholm, where the disease was recognised as psittacosis. Then the blood of both friends was sent by the Swedish doctor to prof. BEDSON in London, who found the complement-fixation reaction with psittacosis antigen strongly positive in both samples. So in the Amsterdam patient the real diagnosis was made half a year afterwards by a Swedish doctor. Four months later we also examined the blood of this patient, and found the complement-fixation reaction still strongly positive (1/128 +++). Most probably the Swedish friend is one of the rare cases in which psittacosis has been transmitted from man to man. In Amsterdam nobody else in the family or among the friends of this patient has been ill with symptoms suggesting psittacosis in severe or light form.

The survey of these cases and two other ones published in 1938 (10), points out, that in Amsterdam among cagebirds the psittacosis virus is endemic or imported, to a yet unknown degree, and gives rise to sporadic cases and small epidemics. There was no severe outbreak, and it seems that special conditions must be fulfilled to attrap the disease. In all the families described above, only once more than one person suffered from psittacosis, no child fell ill. From five children, who were in contact with infected birds, but who had not been ill, the complement-fixation test was negative. In no more than three out of the 11 cases of psittacosis mentioned above, the clinician made the real diagnosis; in all the other patients the disease was supposed to be influenza or an atypical pneumonia.

In order to get an idea about the reliability of the complement-fixation test we examined the blood of several groups of persons.

The sera of 11 patients with atypical pneumonia all gave a negative reaction. The sputum of 7 of them was inoculated into mice, also with negative results.

In four laboratory workers, who more or less often had contact with the virus (two for more than 1½ year daily) no complement fixing antibodies could be demonstrated. From 8 healthy persons out of the families where the sick birds had stayed for a longer or shorter time, only one gave a weakly positive reaction, which disappeared when we used the double hemolytic dose of complement. The other ones were negative.

A few patients who recovered from the disease during an outbreak of psittacosis in 1930 and 1934, could also be tested. Two who had been ill in 1930 gave a negative reaction, but one out of the three patients from 1934 gave a strongly positive reaction (dilution 1/16 +++). Two patients who suffered from psittacosis (from one the virus was isolated) 18 and 19 months ago, both strongly reacted with psittacosis antigen (dilution 1/32 and 1/128 +++). So in judging the value of a positive complement-fixation test it is necessary to keep in mind that though the outcome of the complement-fixation test is very usefull in tracing an epidemic, and also in connection with clinical and epidemiological dates, a history of psittacosis (or perhaps severe influenza!) in the last years makes it nearly worthless for the present illness.

This fact is also demonstrated by the finding of one positive serum among 21 blood samples for the Wassermann test, which had been included in the test as controls. The Wassermann reaction was negative, also the control with normal spleen suspension. The serum was from a woman who suffered from adiposity and sore legs. She had no history of psittacosis or a severe influenza but she did have contact with birds. She often played with a canary which she made bite her under the fingernails. At the autopsy of the bird no pathological findings were made, inoculation of the spleen into mice did not reveal the virus. Five weeks later on we repeated the complement-fixation test, and again it was positive. Only the titer was not as high as in most of the sera from patients. The sera from three of her children and son-in-law were negative, that of the husband gave a weakly positive reaction with the antigen of BEDSON but not with our own (Table II relates some of the complement-fixation tests).

It remains uncertain whether this positive reaction is specific or not. In the first case it may have been a silent infection. Prof. BEDSON who also found a positive reaction in one of his control sera without a history of psittacosis wrote us in one of his letters, that he observed the development of specific antibodies in the absence of any obvious infection in one of his coworkers, who worked with the virus. The observation of GERLACH (8), that the virus can be present in the blood of a patient without symptoms of the disease also points in this direction.

TABLE II.

COMPLEMENT-FIXATION TESTS WITH PSITTACOSIS ANTIGEN.

Donor of serum	time after onset disease when serum taken	serum dilution	antigen E. I.	antigen V. II.	antigen Bedson	control spleen suspension	control saline
v. D.	12 days	1/4	++++	++++	++++	—	—
		1/8	++++	++++	++++	—	—
		1/16	++++	++++	++++	—	—
		1/32	++++	++++	++++		
		1/64	+++	+	+++		
		1/128	+	—	+		
Z.	$3^1/_2$ months	1/4	++++	++++	++++	—	—
		1/8	+++	++++	++++		
		1/16	+++	++++	++++		
		1/32	++	++++	++++		
		1/64	+	+++	+++		
		1/128	—	+	—		
F.	$10^1/_2$ months	1/4	++++	++++	++++	—	—
		1/8	++++	++++	++++		
		1/16	++++	++++	++++		
		1/32	++++	+++	++++		
		1/64	+++	—	+++		
		1/128			+++		
M.	5 years	1/4	+++	++++		—	—
		1/8	+++	++++			
		1/16	+++	+			
		1/32	—	—			
R.	no illness	1/4		++++	++++	—	—
		1/8		++++	++++		
		1/16		+++	++++		
		1/32		+	+		

(In each test controls were included with known positive and negative sera)

Summary.

The psittacosis virus seems to be endemic amongst cage birds in Amsterdam. In animal experiments it is of a low virulence. It gives only occasionally rise to isolated human cases or small outbreaks. The results of animal experiments and complement-fixation tests with material from human cases and infected birds are recorded.

A description is given of a reliable staining method for the virus. Considerations are given about the value of the complement-fixation test.

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(From the Laboratory of the Waterworks, Rotterdam.)

NEW ENRICHMENT METHODS FOR THE CULTIVATION OF *BACTERIUM COLI* AND FAECAL STREPTOCOCCI IN WATER SAMPLES

by

T. FOLPMERS.

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I. NEW ENRICHMENT METHODS FOR *BACTERIUM COLI.*

Difficulties in the detection of *B. coli* in water are numerous. In the Standard Methods for the Examination of Water and Sewage of the American Public Health Association (1920, 1936) the following procedures are given: A. a presumptive test; B. a partially confirmatory test; C. a final confirmatory test for the presence of *B. coli* (the standard lactose broth fermentation test being used).

Gas formation may be due to members of the *B. coli* group, but it not infrequently happens, especially after 48 hours incubation, that it is due either to the symbiotic action of two distinct varieties of bacteria (neither *B. coli*); by certain spore-bearing bacilli (Clostridia); or by *Bac. aerosporus* (1—15) [1].

The efforts of American workers therefore have been devoted to the perfection of media, that will reduce the number of false positives, and they have achieved considerable success (Standard Methods for the Examination of Water, 1936) advocating brilliant green lactose bile broth, to be inoculated in parallel with or to confirm positives from the standard lactose broth, or other typical liquid media having selective growth supporting power. The appendix of the Standard Methods describes these media.

To obviate the above mentioned sources of error KAUFFMANN and SMIT (16) proposed the substitution of peptone by d. glutamic acid in the form of a sodium or ammonium salt.

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[1]) KAUFFMANN describes on page 29 of his dissertation (i.c.) facultative anaerobic Bacilli, called "acid formers", which cause symbiotic fermentation in lactose-peptone with *Granulobacter* and *Clostridium Welchii* in Durham tubes. He for the first time draws attention to the symbiotic fermentation of two spore-forming Bacilli. The fermentation of lactose-peptone in Durham tubes caused by a certain number of cells of *Cl. Welchii* is the easier the larger the quantity of cells of *Bac. subtilis*, which are present to take away the oxygen.

They stated that *B. coli* will grow readily in a medium containing this nitrogenous compound and a sugar in 1 % solution, whilst organisms causing the false presumptive test are unable to do so, though with the addition of 1 % lactose the medium gives somewhat less definite results than those obtained with a peptone-lactose medium at 37° C.

At the same time the author published details of an enrichment medium, in which half of the glutamic acid was replaced by ammonium lactate and lactose by glucose (17).

This medium contains 1 % glucose, 0.5 % glutamic acid neutralised by NaOH, 0.5 % ammonium lactate, 0.5 % NaCl, 0.3 % K_2HPO_4, and tap water pH = 7.0. Cultivation at 45° C. in completely filled stoppered glass bottles. For the examination of surface (river) water, and river water which has undergone various stages of purification (prefiltration and slow sand filtration) the medium is very suitable, being even better for the growth of coliform bacteria and more selective than the EIJKMAN medium (18) which consists of a 1 % solution of glucose and 1 % peptone (Witte). Streptococci do not multiply in contrast to the EIJKMAN medium, in which coli bacteria are often killed by the lactic acid formed by the Streptococci.

The growth of spore-bearing aerobic and anaerobic bacilli (Clostridia), is usually prevented, even after 48 hours cultivation, by lowering the pH of the medium to 6.0. This is important especially in the case of chlorinated waters,

Ammonium lactate is very cheap and is obtainable as an 80 % solution. It is fairly readily utilised, as a source of nitrogen, by *B. coli*, although a better result is obtained by the addition of d. glutamic acid. The mixture of these two nitrogenous compounds has a distinct advantage over peptone, as they are both chemically pure substances of known composition, and difficulties arising from the use of different brands of commercial peptone, of unknown composition, may thus be avoided. During four years employment of this medium I never encountered organisms causing a false presumptive test: Streptococci, *B. proteus* and *Bac. aerosporus* alike failing to grow.

The development of *Aerobacter aerogenes* in the medium is a drawback to the method. In my opinion most of the difficulties connected with the detection of *B. coli*, arise, in the main from two sources of error. Firstly, an incubation temperature of 37° C. is too low; and secondly, peptone, as a source of nitrogen is not sufficiently

selective for *B. coli*, being too favourable to the growth of other bacteria which may cause trouble. The source of nitrogen mentioned above is quite suitable for such a hardy organism as *B. coli*.

A second medium, also very selective for *B. coli* has the following composition: 0.3 % bacto-tryptone, 0.1 % sodium formate, 0.04 % K_2HPO_4, and 0.0001 % crystal violet, dissolved in distilled water, pH = 6.5. Cultivation is performed at 45° C. in completely filled stoppered glass bottles. At 24 and 48 hours gas production is observed and a test for indol formation is made. As a rule they coincide.

Indol formation by *B. coli* is promoted by the addition of sodium formate which acts as a hydrogen donator for the tryptophane molecule, which is hydrogenated and split up to form indol plus alanine. At the same time the formic acid is oxidized to carbon dioxide: a oxidation-reduction reaction. Whilst a trace of sugar (glucose) inhibits indol formation by *B. coli* in a peptone solution, the addition of sodium formate seems, on the contrary, to favour it. At the same time gas production occurs and is easily detected. Sometimes indol production, after 24 hours incubation, is very limited. In such cases the test should be carried out again after 48 hours. Very slight indol production can only be detected by the device of gently heating 3—5 ccm of the coloured culture liquid in a test-tube with a thin supernatant layer of the indol reagent. Indol is very volatile and, by the above means, is caused to accumulate in the reacting zone and is easily demonstrated. The sensitivity of the indol-test applied in this manner is 1 in 2 million ($^1/_2$ γ per ccm).

Fortunately the production of indol by, for instance *B. coli*, is not easily prevented, even though the indol producers are in minority compared with non-indol producers. The addition of crystal violet (1 part per million) is necessary to prevent growth of aerobic spore-bearing bacilli (e.g. *Bac. mesentericus* etc.), which also produce indol. KOVACS's modified technic (19) for the EHRLICH indol test is inapplicable, as crystal violet dissolves readily in amyl alcohol. MICKELSON and WERKMAN (20) found that formate in an acid medium (e.g. pH = 6.5) is rapidly decomposed by *B. coli*, an observation which agrees with my own results. Not only does *B. coli* grow luxuriantly, but in addition *A. aerogenes* and members of the intermediate group. During the summer, at the height of self-purification of river and river-derived waters, members of the *B. proteus* group, which also produce indol and

gas, may at times and for short periods, be present and spoil the results. These can than be corrected if at the same time the above mentioned enrichment medium is employed. During a series of tests extending over a period of three years I have observed that the coli titer found by the two methods was very similar.

For a water-works control and for routine examinations it is, in my opinion, quite useless to determine the various coliform bacteria. The improvement of the coli titer obtained by sedimentation, prefiltration and slow sandfiltration of the raw water should alone be determined, and, in addition the effect of chlorination.

Before I used bacto-tryptone as the source of tryptophane I used, during a period of seven years (21), a tryptophane-peptone solution made from caseine by the action of trypsin (KAHLBAUM).

Here again 0.0001 % crystal violet was added, and incubation at 45° C. was carried out in completely filled glass bottles. The preparation of a tryptophane-containing medium was rendered unnecessary by the introduction of bacto-tryptone by the Difco Company. According to my results the addition of 0.1 % of sodium formate is a distinct improvement of the medium.

II. NEW ENRICHMENT METHODS FOR FAECAL STREPTOCOCCI.

As this group of bacteria has many properties in common with the true lactic acid bacteria the conditions necessary to secure good growth in nutrient media are firstly, the presence of protein, decomposed by a high grade of cleavage (Bacto or Poulenc peptone), and secondly of carbohydrates (sugar, e.g. glucose, lactose, etc.).

Favourable factors are an easily reducible medium and a suitable temperature. The medium should not be buffered as it is desirable that the Streptococci should be able to lower the pH as soon as possible in order to outgrow other bacteria by acidifying the medium.

As lactose is easily utilised by faecal Streptococci (Enterococci), and not so readily by other organisms, this sugar was chosen, in preference, for instance to glucose. Cultivation was carried out in deeply filled test-tubes; for larger quantities of water Erlenmeyer flasks were used (depth of culture medium 10 cm), at a temperature of 45° C.

SHERMAN (22—27) states that, amongst the characteristics of primary differential value in testing for *Streptococcus faecalis*,

is growth at 45° C. From Table 1 (cf. (26)) it may appear that this is a characteristic of all Enterococci.

Acidification of the medium (pH = 7.0) is indicated by the reddening of the litmus solution. The presence of many Streptococci results in a complete reduction, beginning at the bottom of the test-tube. Taking the foregoing factors into consideration, the culture medium adopted was made up as follows: 1 % peptone (Poulenc, Difco or Bacto), 1 % lactose, 0.5 % NaCl, the pH being 7.0. It was tinted with litmus to a deep purple colour.

Cultivation was carried out in test-tubes filled to a depth of 10 cm or in Erlenmeyer flasks, at 45° C.

The presence of faecal Streptococci is readily determined by microscopical examination of the deposit in the tubes or flasks after 20—24 hours incubation. The long chained Streptococci are easy of recognition (31). The examination should be repeated after 48 hours, although the great majority of positives are recognizable after 24 hours.

A second method, highly selective for faecal Streptococci depends on the inhibitory action of 1 % caffeine on other bacteria in a 1 % solution of peptone (29,30). As a result of numerous experiments, it was found that the best culture medium was as follows: 1 % peptone (Poulenc, Difco or Bacto), 1 % caffeine, 0.1 % glucose, 0.3 % Liebig's Beef Extract, 0.5 % NaCl, pH = 7.2.

Incubation at 37° C. in completely filled stoppered glass bottles. The presence of long-chained Streptococci is easily detected, by microscopical examination, after 48 hours incubation. Members of the coli group do not flourish, but, occasionally spore-bearing bacilli prove troublesome (e.g. *Bac. mesentericus, subtilis, mycoides,* etc.). Growth of *Streptococcus faecalis* and *Streptococcus liquefaciens* and other members of the Enterococcus group is luxuriant.

For the purpose of isolation, the ordinary meat-extract gelatine or agar plus 0.5 % glucose may be used. If, however, the lactose-peptone enrichment medium, which gives much less pure cultures, is used, isolation on agar with 0.5 lactose, 1 % peptone, 0.5 % NaCl, 0.2 % K_2HPO_4, with the addition of potassium tellurite 1 : 15000, recommended by Harold (28), may be preferred.

By the use of the above mentioned two methods during the course of a year, the disappearance of faecal Streptococci was demonstrated, following the successive stages of purification (settlement-tanks, prefiltration, and slow sand filtration) as applied in the treatment of surface-water (river water) used for the water supply of Rotterdam.

Streptococci, as well as *B. coli* disappear at all seasons and at all stages of purification, especially in spring, summer and autumn; Streptococci the most quickly (32). They are ingested and destroyed by Amoebae and other Protozoa more especially in stagnant water (settlement-tanks and the water on the slow sandfilters); low temperatures of the water (2° C. and below), when the surface of the settlement-tanks and the slow sandfilters may be covered with ice, appear to inhibit the activity of the Protozoa.

At such times *B. coli* and Streptococci may appear in the filtrate (the finally filtered water), indicating that the situation is bacteriologically dangerous. The presence of Streptococci is an indication of recent faecal contamination; and at such times properly applied and controlled chlorination of the filtered water is essential.

III. PREPARATION OF CULTURE MEDIA.

Media for the cultivation of *B coli*.

a. 0.3 % Bacto-tryptone, 0.04 % K_2HPO_4, 0.1 % HCOONa, 0.0001 % crystal violet, pH = 6.5.

Dissolve in 1800 ccm distilled water 30 grams Bacto-tryptone, 4 grams K_2HPO_4, and 1.5 grams NaCl. Filter through Swedish filter-paper, add ± 24 ccm n.HCl. Sterilise in Koch's steriliser for an hour. Add 50 ccm sterilised 20 % sodium formate solution, 10 ccm sterilised aqueous crystal violet solution (1:1000) and the requisite amount of sterile distilled water to 2000 ccm.

Cultivate in wholly filled stoppered glass bottles at 45° C. Mix with the water to be examined 1:5. For this purpose fill in one fifth of the content with the above mentioned culture medium, and dilute 5 times with the water to be tested, eventually for the smallest dilutions with the requisite amount of sterile water. Test for indol and gas production after 24 and 48 hours.

Indol reagent: 2 grams p.dimethylamino-benzaldehyde in 190 ccm 96 % alcohol and 40 ccm 25 % HCl. Bring the reagent by means of a pipette as a thin layer on part of the culture liquid in a test-tube, and heat the culture liquid gently in the small flame of a Bunsen burner.

b. 1 % glucose, 0.5 % glutamic acid, 0.5 % ammonium lactate, 0.5 % NaCl, 0.3 % K_2HPO_4.

Solution 1: dissolve in 350 ccm hot tap water 12.5 grams d.gluta-

mic acid, 12.5 grams ammonium lactate and 7.5 grams K_2HPO_4. Add ± 16 ccm 5 n.NaOH to bring pH to 7.0. Solution 2: dissolve in 150 ccm tap water 25 grams glucose and 12.5 grams NaCl. Sterilise solutions 1 and 2 apart in Koch's steriliser, and mix them before using.

Cultivate in wholly filled stoppered glass bottles at 45° C. Mix with the water to be examined 1:5. For this purpose fill in one fifth of the content with the above mentioned culture medium, and dilute 5 times with the water to be tested, eventually for the smallest dilutions with the requisite amount of sterile tap water.

Media for the cultivation of faecal Streptococci.

a. 1 % peptone (Poulenc, Difco or Bacto), 1 % lactose, 0.5 % NaCl.

Dissolve in 1000 ccm hot distilled water 20 grams peptone (Poulenc, Difco or Bacto), 20 grams lactose and 10 grams NaCl Filter through Swedish filterpaper, add 9 ccm n.NaOH to make pH = 7.0 and sterilise in Koch's steriliser for an hour. Tint the medium with ± 20 ccm sterile neutral litmus solution to a deep purple colour.

Dilute one time with the water to be tested. Cultivate in deep layers — 10 cm — in test-tubes or in nearly wholly filled Erlenmeyer flasks at 45° C. Examine microscopically the deposit of the tubes and flasks for longchained cocci (Streptococci) after 24 hours repeat after 48 hours.

b. 1 % peptone (Poulenc, Difco or Bacto), 1 % caffeine, 0.3 % Liebig's Beef Extract, 0.5 % NaCl, 0.1 % glucose.

Dissolve in 1000 ccm hot distilled water 20 grams peptone (Poulenc, Difco or Bacto), 20 grams caffeine, 6 grams Liebig's Beef Extract and 10 grams NaCl. Filter through Swedish filterpaper, add ± 16 ccm n.NaOH to make pH = 7.2. Sterilise in Koch's steriliser for an hour, add 10 ccm of a 20 % sterile solution of glucose. Dilute one time with the water to be examined, if necessary with the addition of the requisite amount of sterile water, cultivate in wholly filled stoppered glass bottles at 37° C.

Examine microscopically after 48 hours, sometimes in the indiluted samples after 36 hours. For confirmation the Streptococci may be isolated on lactose-peptone agar with K. tellurite (1:15000), and a Gram-staining may be performed. For routine examination it is not necessary.

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(From the Institute of Preventive Medicine, Leiden.)

MUTUAL CHANCES OF CONTAMINATION WITH TUBERCLE BACILLI FOR MEN AND DOGS

by

J. D. VERLINDE.

(Received June 15, 1939).

It has been found several times, that the bovine tubercle bacillus may be the cause of tuberculosis in men (in the Netherlands i.a. by SPRONCK, DE JONG, BOER, RUYS, VAN DER HOEDEN; cf. VAN DER HOEDEN (10)). As a rule cattle is the source of infection.

Also in domestic animals, as dogs and cats, tuberculosis occurs. At the post mortem examination of 568 dogs, SCHORNAGEL (17) found tuberculosis in 11 of them. 8 Dogs had died of it. Some observations show that during the last years tuberculosis among dogs increases. (MIHAILESCU (11), NIEBERLE and PALLASKE (12)). Thus SCHORNAGEL found in 1914 tuberculosis in 1.9 % of the autopsied dogs, JOEST (cf. NIEBERLE and PALLASKE (12)) in 1917 in 0.83 %, NIEBERLE and PALLASKE in 1931 in 5.2 %. In Stockholm HJÄRRE and HERLITZ (9) found in 1929 tuberculosis in 0.6 %, in 1932 in 5 % of the autopsied dogs.

The following table [1]) shows the type of bacterium:

	Bovin type.	Human type.	Atypical.	Total
SMITH (1905)		1		1
GRIFFITH (1909)		2		2
MALM (1912)	5			5
SCHORNAGEL (1914)	2	4	2	8
ZWICK (1917)	1	1		2
RÖMER (1917)	2			2
JOEST (1917)	1			1
RABINOWITSCH-KEMPNER (1921)	1	16	2	19
PANISSET and VERGE (1922)		2		2
COBETT (1922)	10	7		17

1) Cf. the publications of FELDMANN (6), GRIFFITH (7), HJÄRRE and HERLITZ (9), MIHAILESCU (11), PANISSET and VERGE (13), RABINOWITSCH-KEMPNER (14), RAW (15), SCHORNAGEL (17), STABLEFORTH (18) and of WILSON and LOVELL (19).

Griffith (1928)		1		1
Wilson and Lovell (1928)	1			1
Mihailescu (1929)		8		8
Stableforth (1929)	6	10		16
Feldmann (1930)	1	1		2
Hjärre and Herlitz (1935)	16	21		37
Raw (1935)	1			1
Verlinde (1938)	1	1		2
	48	75	4	127

It has often been found, that in a family suffering from tuberculosis a dog was also infected. (Hjärre and Herlitz (9), O'Connor (4), Carey-Forster (2), Stableforth (18), Escomel (5)).

In some cases we must accept, that dogs have been infected by men, not men by dogs; for instance in the case, mentioned by Escomel, where tuberculous cattle may infect dogs per os (milk).

Danger of infection of men by dogs mostly exists, if the process is located in the lungs of the animal. Pulmonary tuberculosis in dogs nearly always shows a tuberculous broncho-pneumonia with cavities and bronchiectasies. Violent coughing fits occur.

Recently I have observed two cases of tuberculosis in dogs, which are worth mentioning from an epidemiological point of view.

I. The first case was a one year old fox-terrier bought two months before from a farmer. The clinical diagnosis was probably abdominal tuberculosis. At first the owner refused to have the animal killed off. Only after a month we decided to do so. The post mortem examination (Dr. J. Zeldenrust) showed a diffuse, proliferative, partly caseous, tuberculous peritonitis. In the spleen, liver and kidneys some caseating tubercles, and in the lungs some small ones were found. From the caseous areas of liver and spleen, bovine tubercle bacilli were cultivated on the medium of Loewenstein.

Undoubtedly this dog had been infected by cattle, suffering from an open tuberculosis, on the farm. Because the primary-complex was in the abdomen, an infection by way of the intestines must have taken place. An open pulmonary tuberculosis did not yet exist, so there was no direct danger of infection for men. All the same, a tuberculous process was going on in the lungs, which could have become a broncho-pneumonia every moment, connected with coughing and dispersion of sputum, containing tubercle bacilli.

II. A two year old fox-terrier, that had lost weight for some

time, got coughing-fits in the last two weeks, which daily increased in frequency and violence. Sometime before, the daughter of the family, who was very fond of the animal, and even allowed it to sleep in her own bed, had died of an open pulmonary tuberculosis.

At the clinical observation of the dog an extensive affection of the lungs was found. The dog was killed, and the post mortem examination showed a big cavity, which directly communicated with the right main-bronchus. Besides, areas of caseation were found in both lungs.

In the mediastinum, spleen, liver and kidneys caseous tuberculous areas were also found.

Human tubercle bacilli were cultivated from the spleen and the liver.

It may be accepted, that this dog had been infected with human tubercle bacilli, by the patient, suffering from tuberculosis. The primary-complex that undoubtedly existed in the lungs, points to an aerogenic infection.

It is obvious, that such a seriously ill dog, having a large tuberculous cavity in the (right) lung, and suffering during the last weeks from severe coughing-fits, constituted a great danger of infection for the owner and his family.

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(Aus dem Centraalbureau voor Schimmelcultures, Baarn.)

UEBER EINIGE FORMEN VON *VERTICILLIUM DAHLIAE* KLEBAHN

von

F. H. VAN BEYMA THOE KINGMA.

(Eingegangen am 20 Juli 1939).

Aus dem Bakteriologischen Laboratorium der Unilever (Rotterdam) erhielten wir einige Kulturen von Pilzen, welche von feuchtem Packungsmaterial isoliert worden waren. Auffallend war dabei, dass sämtliche Pilze massenhaft Chlamydosporen bildeten, wodurch sie bald eine schwarze Farbe bekamen. Die Erzeugung von ebenfalls massenhaften Konidien an mitunter wirteligen Trägern liess eine Verwandschaft mit den fünf *Verticillium*-Arten: *V. alboatrum* Reinke et Berthold, *V. amaranti* Verona et Ceccarelli, *V. dahliae* Klebahn, *V. ovatum* Berkeley et Jackson und *V. tracheiphilum* Curzi vermuten, weshalb diese Pilze ebenfalls in den Kreis unserer Untersuchungen einbezogen wurden. Schliesslich fügten wir noch *Cephalosporium Serrae* Maffei hinzu, dass bekanntlich ebenfalls Chlamydosporen bildet und auch sonst sich durch seine Eigenschaften den obengenannten Pilzen anzuschliessen scheint und *V. alboatrum* Reinke et Berthold var. *chlamydosporale* Wollenweber, uns von WOLLENWEBER in Berlin freundlichst zur Verfügung gestellt.

Aus dem ausführlichen Schrifttum über diese Verticillien geht hervor, dass viele Autoren sich nicht darüber einig sind, was genau unter *V. alboatrum* zu verstehen ist.

REINKE und BERTHOLD haben nämlich in ihrer ursprünglichen Arbeit (1) von Dauermyzelien gesprochen, die sich aneinander lagern und auf diese Weise braune Zellhaufen bilden, welche man sehr wohl den Namen „Sklerotien" geben könne, wenn sie auch den nach der üblichen Auffassung gewöhnlichen Sklerotien nicht ganz entsprechen. WOLLENWEBER (2) behauptet nun der Pilz, den REINKE und BERTHOLD in Händen gehabt haben sei das häufig vorkommende *V. dahliae* gewesen, welches Sklerotien bildet. Er nimmt denn auch für diesen Pilz den Namen *V. alboatrum* in Anspruch. Das asklerotiale *Verticillium* sei eine seltenere Form, welche von ihm *V. alboatrum* Reinke et Berthold var. *caespitosum* Wollenweber genannt wird. In demselben Sinne äusserten sich u.a. RUDOLPH (3) und VAN DER VEEN (4). Dagegen sind KLEBAHN, VAN DER MEER, BERKELEY und JACKSON der Meinung, das asklerotiale *Verticillium* entspreche

der ursprünglichen Beschreibung, während das „Sklerotien" (Pseudosklerotien oder Mikrosklerotien) bildende *Verticillium* richtig eine neue Art *(V. dahliae)* darstelle. Von BERKELEY (5, 6) ist dann schliesslich eine Arbeit erschienen, worin diese Frage ausführlich untersucht wurde; er hat wahrscheinlich gemacht, dass in der Tat REINKE und BERTHOLD ursprünglich die asklerotiale Form isolierten, sodass man nicht berechtigt sei, bei *V. alboatrum* von „Sklerotien" zu sprechen. Die Ansicht WOLLENWEBER's hat sich denn auch nicht allgemein durchzusetzen vermocht; auch in rezenteren Arbeiten teilt man die Auffassung BERKELEY's und seiner Mitarbeiter. Wir heben hervor, dass das „Centraalbureau voor Schimmelcultures" sich dieser Meinung angeschlossen hat *).

Die hier behandelten Formen sollen jetzt einer kurzen Besprechung unterzogen und, sofern sie neu sind, beschrieben werden. Es hat sich gezeigt, dass die verschiedenen *Verticillium*-Stämme sich am besten in Petrischalen mit Bierwürze-, Kartoffel- und Haferflocken-Agar unterscheiden lassen. Für das Studium der Konidienträger erwiesen sich sterilisierte Kartoffelstücke sehr nützlich, da sie hierauf am ehesten zur Entwicklung gelangten.

Verticillium alboatrum Reinke et Berthold (1).

Stamm VAN DER VEEN, 1930, isoliert von Tomate.
Stamm VAN DER MEER, 1931, isoliert von Kartoffel.

Wie schon oben mitgeteilt, wird als *V. alboatrum* derjenige Wirtelpilz aufgefasst, der meist auf Kartoffel und Tomate vorkommt, keine Pseudosklerotien sondern nur dunkelfarbiges Dauermyzel bildet und Konidienträger erzeugt, welche, besonders in älteren Kulturen an der Basis eine braune Farbe aufweisen. In unseren Kulturen traten nach einiger Zeit nur schwarzbraune bis schwarze Myzelknoten auf, wodurch das Substrat, vor allen Dingen Kartoffelstücke und Bierwürze-Agar, dunkle Streifen bekam. Chlamydosporen wurden nicht gebildet. Wohl wurden in älteren Kulturen dunkelfarbige Hyphen aufgefunden, deren Zellen mehr oder weniger aufgeschwollen waren, echte Chlamydosporen jedoch, als abgerundete dunkle Zellen interkalar in farblosen Hyphen einzeln oder in kurzen Ketten auftretend, wie auch frei im Präparat umherschwimmend, kamen nicht vor. Dieser Pilz sollte daher scharf von allen ähnlichen, Pseudosklerotien oder Chlamydosporen führenden Wirtelpilzen getrennt gehalten werden.

*) Siehe Fussnote S. 757 der Arbeit (6).

Reinkulturen.

Auf Kartoffelstück entsteht anfangs nur weisses wolliges Myzel. Erst allmählich treten am Stück schwarze Stellen auf, aus Dauermyzel bestehend, welche zu schwarzen Streifen auswachsen, während das weisse Myzel einen gelblichen Ton annimmt. Die wirteligen Konidienträger, besonders in älteren Kulturen, sind an der Basis typisch braun gefärbt (Unterschied mit der *V. dahliae*-Gruppe). Die Konidien sind länglich rund bis oval mit 2 bis 3 Oeltröpfchen und meist (4,7 — 8) × (2,3 — 3) μ gross. Nach Klebahn (13) sollen die Konidienträger grössere Abmessungen aufweisen wie diejenigen von *V. dahliae*.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Schale ganz bewachsen mit einer feuchten, glatten Haut mit untiefen, radiären Falten, von weissem, filzigem Myzel überwachsen. Noch keine Bildung von Dauermyzelium Undeutliche Zonenbildung. Unterseite grüngelb. Geruch schwach säuerlich.

Auf Kartoffel-Agar: Schale ganz bewachsen mit einer dünnen Decke aus filzigem, weissem Myzel, mit breiten Zonen wachsend. Noch keine Bildung von Dauermyzelium.

Auf Haferflocken-Agar: Schale ganz bewachsen mit einer dünnen Decke aus filzigem, weissem Myzelium. Noch keine Bildung von Dauermyzelium.

Verticillium alboatrum Reinke et Berthold
var. *chlamydosporale* Wollenweber (2).

Stamm Wollenweber, 1938.

Dieser Pilz, von Sellerie isoliert, wächst anfangs mit weissem Myzel. Die Konidienträger sind an der Basis nicht braun gefärbt, Pseudosklerotien werden nich gebildet, dagegen nach Wollenweber wohl Chlamydosporen. Wir bringen daher den Pilz folgerichtig zu *V. dahliae* unter dem Namen *Verticillium dahliae* Klebahn forma *chlamydosporale* (Wollenweber) van Beyma *n.c.* Im Anschluss hieran möchten wir den von Wollenweber beschriebenen Stamm mit etwas kleineren Konidien, nämlich var. *chlamydosporale* forma *angustum* Wollenweber *) jetzt *V. dahliae* Klebahn forma *angustum* (Wollenweber) van Beyma *n.c.* nennen.

Ein weiterer von Wollenweber beschriebener Stamm, var. *medium* **) bildet ebenfalls Chlamodysporen, wenn auch zögernd

*) l.c. S. 291. **) l.c. S. 289.

Wir haben diesen Pilz, wie auch die forma *angustum* nicht untersucht, doch deutet auch hier wieder die Anwesenheit von Chlamydosporen auf die Verwandtschaft mit *V. dahliae* hin, sodass wir denselben als *V. dahliae* Klebahn forma *medium* (Wollenweber) van Beyma *n.c.* bezeichnen wollen.

Reinkulturen.

Auf Kartoffelstück bildet der Pilz nur weisses, wolliges Myzel, nur allmählich treten an der Glaswand gelbbraune Stellen auf, jedoch hat der Pilz anscheinend die Fähigkeit, Chlamydosporen zu bilden, verloren, da es auch bei fortgesetzter Kultur nicht gelang, dieselben zu erhalten. Nach Wollenweber bildete der Pilz ursprünglich echte Chlamydosporen in grosser Zahl, welche in schwarzen Haufen bei einander lagerten ohne sich in Verbänden anzuordnen. Die Konidien besitzen dieselbe Grösse wie die Grundart, etwa 5,5 × 2,2 μ nach Wollenweber und (5 — 9) × (2 — 2,7) μ (meist (5,3 — 6,3) × (2 — 2,3) μ) nach unseren Messungen.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Schale fast ganz bewachsen mit einer filzig-wolligen, weissen Decke, am Rande einige undeutliche Zonen zeigend. Unterseite gelblich, im Zentrum mit einigen radiären Falten.

Auf Kartoffel-Agar: Schale fast ganz bewachsen mit einer dünnen, farblosen Decke, welche einen spärlichen, weissen Flaum zeigt. Zahlreiche Zonen sind deutlich sichtbar. Rückseite fast farblos.

Auf Haferflocken-Agar: Schale ganz bewachsen mit einer filzigen, flachen, weissen Decke, welche breite Zonen aufweist. Rückseite fast farblos.

Verticillium amaranti Verona et Ceccarelli (7).

Stamm Verona, 1935.

Dieser Pilz, von *Amarantus tricolor* L. isoliert, bildet sowohl terminale wie interkalare Ketten von Chlamydosporen. Er ist daher nicht, wie Goidanich (8) meint, dem *V. alboatrum* gleich zu stellen, sondern gehört in die *V. dahliae*-Gruppe. Die Grösse der Konidien wird als (4,8 — 6,4) × (3 — 3,2) μ angegeben, die der Chlamydosporen als 6,4 × 9,6 μ, Nach unseren Messungen sind die Konidien (3,7 — 6,7) × (2,3 — 4,3) μ, meist (4,3 — 4,7) × (2,3 — 2,7) μ gross, mit zwei Oeltropfen. Die Eigenschaften und das Wachstum des Pilzes auf verschiedenen Nährböden stimmen fast vollkommen mit *V. Serrae*

(Maffei) van Beyma überein, weshalb wir diese Pilze als Synonyme betrachten (Siehe S. 40).

Reinkulturen.

Auf Röhrchen nach 1 Monat:

Auf Kartoffelstück: das Stück ganz bewachsen mit weissem Myzel, bestehend aus zahlreichen Hyphenbündeln. Fast keine Bildung von Chlamydosporen, das Stück ist nur oben etwas schwärzlich.

Auf Haferflocken-Agar: oben weisses, filziges Myzel, unten ein grauschwarzes Stroma infolge der Bildung von Chlamydosporen. Rückseite und Agar farblos.

Auf Maismehl-Agar: weisses, filziges Myzel, an der Glaswand eine schwarze Linie bildend. Rückseite und Agar farblos.

Auf Kartoffel-Agar: schwarzes Stroma, von kurzem Myzel überwachsen. An der Glaswand eine intensiv schwarze Linie. Rückseite schwärzlich, Agar farblos.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Kolonie 4 cm im Durchmesser, bestehend aus einer weissen filzigen bis flachwolligen Decke, zum Teil zottig durch zahlreiche aufstehende Hyphenbündel. Rand scharf. Noch keine Bildung von Chlamydosporen. Unterseite hellgelb bis leicht orangegelb.

Auf Kartoffel-Agar: Schale fast ganz bewachsen mit einem kurzen, weissen Flaum, in den zentralen Partien mit zahlreichen, schmalen Zonen, durch bereits eingetretene Chlamydosporenbildung dunkel gefärbt.

Auf Haferflocken-Agar: Schale ganz bewachsen mit einem kurzen, weissen Flaum mit zahlreichen schmalen Zonen. Noch keine Bildung von Chlamydosporen.

Verticillium cinerescens Wollenweber (2).

Dieser Pilz von welkekranken Nelken, *Dianthus caryophyllus*, isoliert, wurde nur deshalb mit herangezogen, weil Wollenweber denselben in seiner *Verticillium*-Arbeit als neue Art beschreibt. Der Autor hat ihn jedoch nur vorläufig zur Gattung *Verticillium* gestellt. In der Tat ist es auch gar kein *Verticillium* wie schon aus der büscheligen Art, wie die Konidienträger am Myzel sitzen, hervorgeht. Nach genauer Untersuchung stellte sich heraus, dass der Pilz zur Gattung *Phialophora* Medlar gehört (9). Identisch mit dieser Gattung ist *Cadophora* von Lagerberg, Lundberg und Melin 1927 ausführlich beschrieben (10).

Conant (11) wies dann auf die Priorität des Gattungsnamens

Phialophora Thaxter hin. Die Konidien entstehen so wie es von mir beschrieben wurde (12). Besonders in älteren Kulturen sind nach Abschwemmung der Konidien die Konidienträger mit ihren typischen „Schnabeln" deutlich zu sehen. Dieser Pilz soll deshalb besser *Phialophora cinerescens* (Wollenweber) van Beyma *n c* genannt werden.

Verticillium dahliae Klebahn (13).

Stamm Buisman, 1927, von Ulme.
Stamm C.B.S., 1930, von Linde.
Stamm van der Meer, 1927, von *Erigeron Canadensis.*
Stamm Stat. de Path. Vég. de Brive, 1927, von Artischocke.
Stamm van der Veer, 1930, von Phlox.

Obige Stämme wurden miteinander sowohl auf Kartoffel- wie auf Haferflocken-Agar in Petrischalen nach 1 Monat verglichen. Mikroskopisch ähneln die wirteligen Konidienträger denen von *V alboatrum,* nur dass sie an der Basis nicht braun werden. Die Konidien besitzen dieselbe Grösse und Gestalt wie diejenigen von *V. alboatrum.* Die Pseudosklerotien, welche bei *V. alboatrum* fehlen, stellen grosse, braunschwarze Gebilde dar (bis 50 μ), welche am Rande von Agar-Kulturen schon mit dem unbewaffneten Auge als zahlreiche Pünktchen wahrgenommen werden können.

Was die Bildung von Pseudosklerotien und die dadurch hervorgerufene Schwärzung der Nährböden anbetrifft, stellte sich heraus, dass es alle möglichen Uebergänge gibt, sodass es unmöglich erscheint, von diesen Stämmen einzelne als besondere Arten abzutrennen. Wenn wir die als Synonyme betrachteten Arten *V. tracheiphilum* und *V. ovatum* denn auch an dieser Stelle hinzuziehen, ergibt sich folgendes Bild:

Reinkulturen in Petrischalen nach 1 Monat:.

Auf Kartoffel-Agar: im allgemeinen wächst *V. dahliae* nicht sehr gut auf Kartoffel-Agar, wenigstens werden die Pseudosklerotien nur zögernd gebildet. Bei den Stämmen Buisman, Brive, van der Meer und van der Veer entstehen nur flache, häutige Kolonien, farblos, mit zarten Zonen. Nur im Zentrum bilden sich kleine, durch Pseudosklerotien schwarze Stellen, wo die Zonen denn auch deutlicher hervortreten. *V. tracheiphilum* bildet etwas mehr weisses, wolliges Myzel. Nur *V. ovatum,* und in geringerem Masze auch Stamm C.B.S. besitzen die Fähigkeit in kurzer Zeit grössere schwarze Stellen im Zentrum zu bilden.

Auf Haferflocken-Agar: Die Stämme van der Meer, van der Veen und Brive bilden flache Kolonien, mit undeutlichen Zonen

und mit einem Ueberwuchs von weissem, filzigem Myzel. Unterseite farblos. Pseudosklerotien werden nur spärlich gebildet. Bei Stamm Brive fällt noch das kreisförmig um das Impfstück herum in eigentümlichen grösseren und kleineren flachen Feldern auftretende filzige Luftmyzel auf. *V. ovatum* erzeugt im Zentrum etwas mehr Pseudosklerotien, welche in radiären Streifen angeordnet sind. Dagegen erzeugen die Stämme C.B.S. und Buisman Pseudosklerotien in grossen Massen, sodass die Kulturen dadurch geschwärzt sind, die schönen regelmässigen Zonen treten deutlich hervor und sind von einem spärlichen, weissen Flaum überwachsen. Die Unterseite ist schwärzlich mit deutlicher Zonierung.

Verticillium ovatum Berkeley et Jackson (5).

Dieses *Verticillium* wurde von Berkeley und Jackson als Erreger einer Rubus-Verticilliose in Canada festgestellt und als neue Art beschrieben. Nach den Autoren soll der Pilz Unterschiede im Wachstum und in der Bildung von Pseudosklerotien mit *V. dahliae* aufweisen. Nach Wollenweber ist der Pilz jedoch als *V. dahliae* zu betrachten. Er wurde von uns untersucht, auf verschiedenen Nährböden gezüchtet und mit den *V. dahliae*-Stämmen verglichen. Zweifellos muss der Pilz in die *V. dahliae*-Gruppe untergebracht werden. Zwar erzeugt er auf Kartoffel-Agar eine dunklere Decke als die anderen Stämme, doch muss bei den vielen Uebergängen, welche es in dieser Beziehung gibt, eine Aufrechterhaltung als besondere Art nicht durchführbar erachtet werden.

Reinkulturen.

In Petrischalen nach 1 Monat:

Auf Kartoffel-Agar: Kolonie 6 cm im Durchmesser, flach, häutig, in den zentralen Partien schwarz durch die Bildung von Pseudosklerotien in Zonen, teilweise von weissem, flockigem Myzel überwachsen. Nach dem Rande hin farblos. Unterseite im Zentrum schwarz, die Zonen treten deutlich hervor.

Auf Haferflocken-Agar: Schale ganz bewachsen. Kolonie flach, häutig, farblos, nur im Zentrum eine geringe Entwicklung von Pseudosklerotien, in radiären Streifen angeordnet. Unterseite farblos.

Cephalosporium Serrae Maffei (14).

Stamm Pollacci, 1930.

Dieser Pilz wurde von Serra (Parma) aus dem Auge eines männlichen Patienten isoliert und von Maffei 1929 als neue Art beschrieben. Einige Jahre später erschien eine Mitteilung von Focosi (15),

worin er diesen Pilz mit *Cephalosporium Stühmeri* Schmidt et van Beyma meinte identifizieren zu können (19). In Erwiderung hierauf teilte VAN BEYMA THOE KINGMA (16) in einer kurzen Notiz die Unterschiede zwischen beide Pilze mit, welche er als gute Arten betrachten müsse. Indem letztgenannte Mitteilung mehr die Artgleichheit beider Pilze zu bestreiten beabsichtigte als die systematische Stellung der neuen *Cephalosporium*-Art zu untersuchen, wurde der MAFFEI'sche Pilz kulturell nicht bis in allen Einzelheiten studiert. Jedoch war uns von jeher eine gewisse Aehnlichkeit mit *V. dahliae* nicht entgangen und als nun im Kreise dieser Untersuchungen die Entwicklung des Pilzes auf verschiedenen Nährböden näher geprüft wurde, ergab sich, dass dieses *Cephalosporium* in seiner höchsten Entwicklungsform ein *Verticillium* darstellt. Diesen Pilz, der also dem *V. dahliae* nahesteht, werden wir deshalb als *Verticillium Serrae* (Maffei) van Beyma *n.c.* bezeichnen.

Reinkulturen.

Auf Kartoffelstück entwickelt sich meist eine feuchte, glatte, farblose Haut, welche jedoch oben im Röhrchen in eine grauschwarze Decke übergeht, von einem kurzen Flaum überzogen. In dem spärlichen Luftmyzel findet man Konidienträger mit ausgebildeten Wirteln. Im allgemeinen steht die Verzweigung jedoch hinter die des *V. alboatrum* und *V. dahliae* zurück. Die Chlamydosporen, welche sowohl terminal wie interkalar in Ketten vorkommen, erscheinen oft doppelt konturiert, meist enthalten sie ein oder mehrere Oeltröpfchen. Die Konidien besitzen eine mehr längliche Gestalt wie bei *V. alboatrum*, auch sind sie etwas schmaler, meist (4,7 — 6) × (1,7 — 2) μ mit zwei Oeltröpfchen.

Nach 1 Monat ist das Stück zum grössten Teil schwarz geworden, mit stellenweise weissen Myzelflächen dazwischen. An der Glaswand ist die schwarze Farbe besonders ausgeprägt.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: eine grosse Kolonie, 4 cm im Durchmesser, bestehend aus filzig-wolligen, aufstehenden Hyphenbündeln, sodass sie mehr oder weniger zottig aussieht. Am Rande der Kolonie ein flacher, farbloser Saum, welche feine Zonen zeigt. Rand scharf. Unterseite schmutzig grüngelb.

Auf Kartoffel-Agar: Kolonie 6,5 cm, bestehend aus einer flachen, farblosen Decke, welche nach dem Zentrum hin immer mehr flaumig und grau wird, radiär gestreift und mit deutlichen, feinen Zonen. Schon zahlreiche Chlamydosporen haben sich gebildet, welche die Kultur dunkel färben. Unterseite grauschwarz im Zentrum, sonst farblos.

Auf Haferflocken-Agar: Schale ganz bewachsen mit einer filzigen, flachen, weissen Decke, welche zahlreiche Zonen aufweist, ähnlich wie die forma *chlamydosporale* von Wollenweber. Der Unterschied mit diesem Pilze ist auf Bierwürze-Agar jedoch ziemlich gross.

Verticillium tracheiphilum Curzi (17, 18).

Stamm Pollacci, 1928.

Dieser Pilz von *Capsicum annuum* isoliert, bildet in der Kultur zahlreiche Pseudosklerotien. Die Grösse der Konidien beträgt nach unseren Messungen (4 — 7) × (2,3 — 3,3) μ, meist (4 — 4,3) × (2,7 — 3) μ Sie enthalten zwei Oeltröpfchen. Dieser Pilz weist eine derartig grosse Aehnlichkeit mit *V. dahliae* auf, dass wir ihn als Synonym betrachten. Wir haben den Pilz nämlich in Reinkulturen verglichen mit sämtlichen Stämmen von *V. dahliae* aus der Sammlung des C.B.S. *V. tracheiphilum* kommt im Wachstum am meisten mit den Stämmen van de Veer, van der Meer und Brive überein. Wie diese wächst der Curzi'sche Pilz anfangs mit weissem, filzigem Myzel, in undeutlichen Zonen, während erst nach mehreren Wochen Pseudosklerotien gebildet werden. Die Stämme Buisman und C.B.S. dagegen wachsen vom Anfang an in deutlichen, schwärzlichen Zonen infolge der Bildung von Pseudosklerotien, denen ein flaches, flaumiges, weisses Myzel vorangeht.

Reinkulturen.

Auf Röhrchen nach 1 Monat:

Auf Kartoffelstück: das Stück ganz bewachsen mit einem wolligen, weissen Myzel, dass fast das ganze Röhrchen füllt. Nach unten wird die Myzeldecke durch einen schwarzen Stroma-Rand abgegrenzt.

Auf Haferflocken-Agar: wolliges, weisses Myzel, an der Glaswand eine schwarze Linie bildend.

Auf Maismehl-Agar: weisses, wolliges Myzel. An der Rückseite ist eine schwarze Stelle sichtbar, wo sich ein Stroma entwickelt, sonst farblos.

Auf Kartoffel-Agar: eine flachwollige bis filzige, weisse Decke. An der Rückseite ist ein schwarzes Stroma sichtbar.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Kolonie 4 cm, bestehend aus einer weissen, wolligen Decke mit schmalem, farblosem, fast submersem Rande. Keine Pseudosklerotien. Unterseite gelblich.

Auf Kartoffel-Agar: Schale ganz bewachsen mit einer dünnen, farblosen Decke, in den zentralen Partien schwärzlich durch

die Bildung zahlreicher Pseudosklerotien, überwachsen von einem flachwolligen, weissen Myzel. Um das Zentrum herum zahlreiche Zonen, welche im geschwärzten Teil am deutlichsten hervortreten. Unterseite nur im Zentrum schwarz und deutlich zoniert, sonst farblos.

Auf Haferflocken-Agar: Schale ganz bewachsen mit einer dünnen, filzigwolligen, weissen Myzeldecke, im Zentrum anfangend etwas dunkler zu werden infolge der Bildung von Pseudosklerotien. Die Zonen sind nur undeutlich zu sehen. Unterseite farblos, nur im Zentrum einige dunkle Zonen.

Bei den nun folgenden Stämmen von Unilever (Rotterdam) ist die Bildung von wirteligen Konidienträgern selten. Dieses kommt auch bei bestimmten Stämmen von den genannten Verticillien vor. In den meisten Fällen bildet sich nur ein einziger Wirtel am Scheitel des Trägers, aus 3—5 Sterigmen bestehend, nur nach mehrmaligem Ueberimpfen auf geeigneten Nährböden wie Kartoffelstücken gelingt es, stärker verzweigte Konidienträger zu erhalten. Im übrigen stimmen diese Pilze so stark mit *V. dahliae* überein, dass wir sie als Formen desselben betrachten. Die Gestalt und Grösse der Konidien, die Bildung von massenhaften Chlamydosporen, welche sich in besonderen Fällen zu Pseudosklerotien verdichten können und schliesslich das Wachstum auf den verschiedenen Nährböden, es sind alle Merkmale wie sie auch bei den verschiedenen Stämmen von *V. dahliae* gefunden werden.

Wir lassen die Beschreibung dieser Formen hier folgen.

Verticillium dahliae Klebahn forma *zonatum* n.f.

Stamm No. 1, Unilever, 1937.

Dieser Pilz ähnelt dem *V. dahliae* Stamm C.B.S. am meisten. Er wächst auf allen Nährböden üppig und rasch unter Bildung einer schwarzen Decke, welche jedoch keine Pseudosklerotien sondern Chlamydosporen in Massen enthält. Er zeichnet sich aus durch schöne Zonenbildung, besonders auf Haferflocken- und Kartoffel-Agar in Petrischalen.

Reinkulturen.

Auf Kartoffelstück bildet sich eine schwarze, runzelige Haut mit nur wenig Luftmyzel, das bald verschwindet. Die Konidien besitzen dieselbe Grösse und Gestalt wie die des *V. alboatrum*. Die schwarzbraunen Chlamydosporen, welche im allgemeinen von geringerer Grösse sind wie diejenigen von *V. dahliae* wachsen interkalar einzeln in gewissen Abständen in den Hyphen oder bilden

lange, dunkelfarbige Ketten. Meist sind die Hyphen durch bündelförmige Zusammenlegung wieder zu Strängen vereint.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Kolonie 3 cm im Durchmesser, bestehend aus einer faltigen Decke mit grauweissem, wolligem Ueberwuchs. Im Zentrum etwas erhaben und daselbst fast schwarz. Um die Kolonie herum ein farbloser Saum, 2 mm breit. Unterseite schwarz mit kleinen, radiären Falten.

Auf Kartoffel-Agar: Kolonie 7 cm im Durchmesser, bestehend aus einer flachen, glatten, häutigen Decke, fast ohne Luftmyzel, schwarz, in schönen konzentrischen Zonen. Unterseite schwarz.

Auf Haferflocken-Agar: Kolonie 5 cm, bestehend aus einer tiefschwarzen Decke mit zahlreichen schönen, feinen Zonen, von einem zarten Flaum überwachsen. Der Rand zeigt einige radiäre Streifen. Unterseite schwarz mit violettem Stiche und deutlicher Zonierung.

Lateinische Beschreibung.

A typo differt rapida formatione chlamydosporarum qua brevi tempore media fusca fiunt; formatione pulchrarum zonarum in agare carbonhydratico.

Verticillium dahliae Klebahn forma *cerebriforme* n.f.

Stamm No. 4, Unilever, 1937.

Von diesem Pilze erhielten wir noch drei weitere Stämme. Am meisten auffallend ist sowohl bei No. 4 wie bei der weiter unten beschriebenen Form C 1 das begrenzte Wachstum auf Bierwürze-Agar in Petrischalen, worüber unten nähere Angaben gemacht werden. Aber auch hier nur selten eine vollständige Wirtelbildung wie bei *V. dahliae*. Die Chlamydosporen entstehen sowohl terminal wie interkalar typisch in langen Ketten. Die terminalen Ketten, am Ende eines kurzen, hyalinen Hyphenzweiges, können sich zu kleinen Pseudosklerotien verdichten. Die Konidien sind eiförmig, mitunter einerseits etwas zugespitzt, meist (3,7 — 5,3) × (2,3 — 3,0) μ. Oeltröpfchen fehlen.

Reinkulturen.

Auf Kartoffelstück entsteht eine dicke, schwarze Kruste, teilweise von einem grauen Flaum überzogen. Luftmyzel fehlt fast gänzlich.

In Petrischalen nach 1 Monat:

Auf Bierwürze-Agar: Kolonie 1 cm im Durchmesser, mit stark begrenztem Wachstum. Die Decke ist knorpelig, am Rande etwas in den Agar hineingewachsen, mausgrau mit braunem Stiche, im Zentrum erhaben mit einigen Windungen. Unterseite violettschwarz.

Auf Kartoffel-Agar: Kolonie 2 cm im Durchmesser, bestehend aus einer flachen Decke mit radiären Falten, schwarzgrau, im Zentrum erhaben mit tiefen radiären Falten, am Rande mit feinen Zonen. Um die Kolonie herum ein 2—3 mm breiter, farbloser Saum. Unterseite violett-schwärzlich.

Auf Haferflocken-Agar: Kolonie 2 cm im Durchmesser, bestehend aus einer flachen, im Zentrum mehr knorpeligen Decke, hellgrau. Rand scharf. Unterseite violett-rötlich.

Lateinische Beschreibung.

A typo differt valde determinata cerebriformi crescentia in agaro hordei madidi (Bierwürze) et formatione chlamydosporarum longis catenis.

Verticillium dahliae Klebahn forma *restrictum* n.f.

Stamm C 1, Unilever, 1937.

Dieser Pilz stimmt mit No. 4 überein in dem begrenzten Wachstum auf Agar-Nährböden, weicht aber sowohl in der Farbe, wie in der Gestalt, nicht aber in der Grösse der Konidien und Chlamydosporen von demselben ab. Während erstgenannter auf Bierwürze bald eine mausgraue Farbe annimmt, bleibt C 1 längere Zeit kremfarben, um im Alter ein wenig graugrün zu werden. Die Chlamydosporen wachsen weniger in regelmässigen Ketten, sondern es entstehen meist unregelmässige, dunkel gefärbte Gebilde, woraus mitunter hyaline Konidienträger hervorgehen. Die Konidienträger bilden nur selten Wirtel. Die Konidien sind kugelig bis subglobos, meist (3 — 3,3) × (2,3 — 2,7) μ gross und hyalin.

Reinkulturen.

Auf Kartoffelstück wächst der Pilz unter Bildung dicker, halbkugeliger Myzelmassen, welche anfangs weiss, später teilweise graugrün werden. An der Glaswand nur vereinzelt einige dunklere Stellen.

In Petrischalen nach 1 Monat:

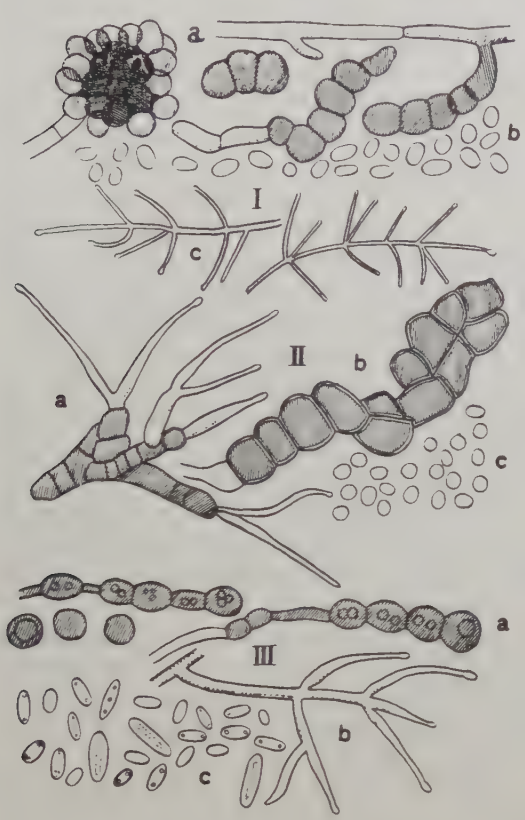
Auf Bierwürze-Agar: Kolonie 1 cm im Durchmesser, mit einer knorpeligen etwas flaumigen, kremfarbenen Haut, am Rande etwas in den Agar hineingewachsen. Unterseite rötlich-gelblich, hohl.

Auf Kartoffel-Agar: Kolonie 1,5 cm im Durchmesser, im Zentrum etwas erhaben und daselbst hellgrau, nach dem Rande hin fast weiss. Farbloser Saum 2 mm. Unterseite violett-schwärzlich im Zentrum, sonst farblos.

Auf Haferflocken-Agar: Kolonie 2 cm im Durchmesser, flach, im Zentrum etwas erhaben, hellgrau bis kremfarben und filzig-wollig, am Rande flach, flaumig, hellgrau. Unterseite im Zentrum schwärzlich, sonst gelblich.

Lateinische Beschreibung.

A typo differt valde determinata crescentia in agaro hordei madidi (Bierwürze) atque rotundis vel subglobosis conidiis.



I. *Verticillium dahliae* f. *cerebriforme.*

a. Chlamydosporen, 750 ×
b. Konidien, 750 ×
c. Konidienträger, 245 ×

II. *Verticillium dahliae* f. *restrictum.*

a. Konidienträger, 750 ×
b. Chlamydosporen, 750 ×
c. Konidien, 750 ×

III. *Verticillium dahliae* f. *zonatum.*

a. Chlamydosporen, 750 ×
b. Konidienträger, 750 ×
c. Konidien, 750 ×

Zusammenfassung.

Es wurden einige mit *Verticillium dahliae* Klebahn verwandte Formen, isoliert von feuchtem Packungsmaterial mit der Grundart und einigen anderen, nahestehenden Vertretern der Gattung *Verticillium* auf verschiedenen Nährböden miteinander verglichen und beschrieben. Die beide Arten *V. alboatrum* und *V. dahliae* sind scharf von einander abzugrenzen. *V. alboatrum* bildet nur dunkelfarbiges Dauermyzel, welches sich zu schwarzen Myzelknoten verdichten kann, ausserdem sind die älteren Konidienträger an der Basis braungefärbt, während Chlamydosporen und Pseudosklerotien fehlen. *V. dahliae* dagegen erzeugt zahlreiche Pseudosklerotien. In

der Regel nehmen die Myzeldecken hier viel schneller eine schwarze Farbe an wie bei *V. alboatrum*, das meist in Reinkulturen längere Zeit hindurch nur weisse Decken bildet. Die Befürchtung WOLLENWEBER's, die Auffassung seiner Gegner würde „das *V. alboatrum* zu einer Rarität stempeln" *), trifft in der Tat zu. *V. alboatrum* ist eine Art für sich, welche verhältnissmässig selten gefunden wird, hauptsächlich auf Kartoffel und Tomate. Dagegen kommt *V. dahliae* allgemein in der Natur auf verschiedenen Pflanzen vor. Ausserdem scheint, wie aus Obigem hervorgeht, es eine ganze Reihe von Organismen zu geben, welche dem *V. dahliae* nahestehen, einmal durch die Bildung von Chlamydosporen, welche sich manchmal zu Pseudosklerotien verdichten, und weiter durch die Verzweigung ihrer, nicht an der Basis braun gefärbter Konidienträger. Diese erreichen zwar selten mehrfach wirtelige Verzweigung trotz fortgesetzter Uebertragung auf geeigneten Nährböden, jedoch dürfen diese Pilze auf keinen Fall der Gattung *Cephalosporium* zugerechnet werden, von der sie sich schon durch die Bildung einfacher Wirtel entfernen. Aus diesem Grunde wurde auch *C. Serrae* zur Gattung *Verticillium* gebracht unter dem Namen *Verticillium dahliae* (Maffei) van Beyma *n.c.*

Nachdem der Name *V. alboatrum* im Sinne WOLLENWEBER's wieder abgeändert ist in *V. dahliae* Klebahn, müssen folgerichtig auch die Varietäten WOLLENWEBER's als *formae* zur *dahliae*-Gruppe gebracht werden. Die var. *chlamydosporale* Wollenweber ist demnach *V. dahliae* Klebahn forma *chlamydosporale* (Wollenweber) van Beyma *n.c.* zu benennen. Ebenso sollen die Varietäten *V. alboatrum* Reinke et Berthold var. *chlamydosporale* forma *angustum* Wollenweber und *V. alboatrum* Reinke et Berthold var. *medium* Wollenweber heissen: *V. dahliae* Klebahn forma *angustum* (Wollenweber) van Beyma *n.c.* und *V. dahliae* Klebahn forma *medium* (Wollenweber) van Beyma *n.c.* Anschliessend an BERKELEY, MADDEN und WILLISON verstehen wir unter den von WOLLENWEBER *Verticillium alboatrum* Reinke et Berthold var. *caespitosum* Wollenweber genannten Pilz die Grundart *Verticillium alboatrum* Reinke et Berthold, welche keine Pseudosklerotien bildet im Gegensatz zu *Verticillium dahliae* Klebahn. *Verticillium amaranti* Verona et Ceccarelli ist *Verticillium Serrae* (Maffei) van Beyma, *Verticillium tracheiphilum* Curzi ist *Verticillium dahliae* Klebahn, ebenso *Verticillium ovatum* Berkeley et Jackson.

Schliesslich wurden als neue Formen von *Verticillium dahliae* beschrieben: *V. dahliae* Klebahn forma *zonatum*, *V. dahliae* Klebahn forma *cerebriforme* und *V. dahliae* Klebahn forma *restrictum*. *Ver-*

*) l.c. S. 275.

ticillium dahliae ist also eine „Gruppen-Art" wie man sie bei den Penicillien kennt: sie besteht aus verschiedenen Typen oder Formen. Zur Art-Benennung dieser kleine Einheiten liegt kein Grund vor.

Das von Wollenweber beschriebene *V. cinerescens* ist kein *Verticillium* sondern gehört zur Gattung *Phialophora* und muss deshalb *Phialophora cinerescens* (Wollenweber) van Beyma *n.c.* genannt werden.

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(Laboratoire d'Hygiène, de Bactériologie et d'Hygiène Tropicale
de l'Université de l'État à Leiden.)

SUR LES FERMENTS PROTÉOLYTIQUES DU *VIBRIO CHOLERAE* ET DU *VIBRIO EL TOR*

par

H. BEEUWKES.

(Reçu le 27 juillet 1939).

On sait que le *Vibrio cholerae* ensemencé sur une plaque au sang, est capable de produire, après quelque temps, une zone verte à l'endroit de la culture. Cette zone est complètement transparente et nettement délimitée. Très vraisemblablement un ferment hémodigestif est responsable de ce phénomène.

Après repiquages répétés de gélose sur gélose, ce pouvoir diminue d'intensité, pour finalement disparaître. Pourtant quelques souches conservent plus longtemps leurs propriétés hémodigestives, tandis que des cultures, qui en sont complètement dépourvues, peuvent provoquer l'apparition passagère d'une zone verte sur la plaque. Simultanément, le pouvoir de scinder la gélatine diminue d'intensité, quoiqu'il continue d'exister chez la plupart des souches. La culture sur certains milieux permet de mettre en évidence des variations d'activité de ce ferment.

Les propriétés des deux pouvoirs se ressemblent. La variabilité aussi offre des points de ressemblance. C'est pourquoi il n'est pas étonnant que la liquéfaction de la gélatine et l'hémodigestion soient considérées comme des manifestations d'un même ferment (VAN LOGHEM (5)). Mais, ainsi que BOTMAN (3) le fait remarquer, ce parallélisme de différentes propriétés n'indique pas nécessairement que les deux phénomènes relèvent de l'activité d'un seul et même ferment. Ce chercheur interprête la chose en admettant que les mêmes influences font subir en général les mêmes changements aux propriétés fermentatives.

Le but de cette recherche est de fournir une contribution à cette dernière conception.

Dans une communication récente, BERNARD et ses collaborateurs (2) communiquent qu'ils purent démontrer la présence d'une substance pourvue de propriétés hémodigestives dans des milieux solides et liquides ensemencés d'une souche de choléra hémodigestive. A côté

de l'action protéolytique, cette substance avait le pouvoir de décomposer le sang jusqu'à l'apparition d'hématine, en passant par un stade pendant lequel il se trouvait de l'oxy-hémoglobine dans l'éprouvette. Une modification de technique me permit de même de retrouver, dans des extraits de gélose, sur lequel des vibrions cholériques hémodigestifs s'étaient développés, une protéase ayant les propriétés décrites par BERNARD.

Comment se comporte maintenant, à l'égard de la gélatine et du sang, la protéase originaire d'une souche de choléra non hémodigestive?

Deux souches de cholera, à savoir Ch. 86 et Ch. 91, servirent à cette recherche. Les deux souches étaient fort bien agglutinées par un sérum anti-cholérique. Ch. 86, ensemencé sur une plaque avec 10 % de sang de chèvre, ne montra aucune zone, même après 4 jours de culture. Ch. 91 provoqua la formation, après 3 jours de culture, d'une zone verte, claire et nettement délimitée. La gélatine fut liquéfiée en même temps à peu près.

Une culture de chaque souche sur gélose, âgée de 24 heures, fut préparée dans deux flacons de Roux contenant environ 200 cc de gélose nutritive à 3 %. Après 4 jours de culture à 36°5 C., les bactéries furent enlevées au moyen d'eau distillée stérile, la masse gélosée fut broyée, et 200 cc d'eau distillée stérile furent ajoutés à chaque flacon. Deux flacons non ensemencés servirent de contrôle. Le contenu des flacons fut filtré sur ouate stérile après une semaine de séjour à la glacière; 150 cc de ce filtrat furent précipités par 750 cc d'acétone-alcool à 97 % ana. Cette méthode de précipitation fut mise au point par KAYSER (4) pour l'isolement des protéines sériques. Ma thèse (1) rapporte des données complémentaires à ce sujet.

Après dessiccation à 37° C., le précipité fut dissous dans 15 cc de solution de NaCl à 0,9 %. De la même manière, 250 cc acétone-alcool à 96 % ana servirent à précipiter 50 cc de filtrat provenant de gélose non ensemencée; et, après dessiccation, le précipité fut dissous dans 8 cc de solution de NaCl à 0,9 %. L'action sur la gélatine fut examinée par l'addition de 1 cc du précipité dissous à 2 cc de gélatine à 5 % ayant un pH de 7.5, puis par une incubation de 2 heures à 36°5 C. suivie du séjour des tubes pendant 1 heure à la dissous dans 8 cc de solution de NaCl à 0,9 %. L'action sur la gélatine dans l'eau distillée, en portant le pH à 7.5, et en chauffant ensuite pendant 10 minutes à 100° C. Le résultat peut se lire dans e tableau I.

TABLEAU I.

INFLUENCE DE LA PROTÉASE CHOLÉRIQUES SUR LA GÉLATINE.

Numéro de la souche.	Quantité de précipité dissous.	Gélatine à 5 %.	2 heures à 36°5 C. et ensuite 1 heure à la glacière.
Ch 91	1 cc +	2 cc	liquéfaction
Ch 86	1 cc +	2 cc	liquéfaction
Contrôles:			
Extrait de gélose non-ensemencée	1 cc +	2 cc	coagulation
		2 cc	coagulation

On voit que les précipités, provenant d'une gélose nutritive ensemencés au moyen de deux souches de choléra, peuvent liquéfier la gélatine après 2 heures de contact à 36°5 C. Le précipité de l'extrait de gélose non ensemencée n'a pas d'action protéolytique.

L'influence de différents précipités sur sang de chèvre fut ensuite examinée. Les résultats de ces épreuves se lisent au tableau II.

TABLEAU II.

INFLUENCE DE LA PROTÉASE CHOLÉRIQUE SUR SANG DE CHÈVRE.

Numéro de la souche.	Précipité de l'extrait de gélose.	Emulsion de sang de chèvre à 5 %.	Après 24 heures à 36°5 C.	Après 48 heures à 36°5 C.
Ch 91	5 cc +	1 cc	Absence d'hémolyse	Couleur brune verte. Hématine alcaline.
Ch 91	5 cc +	1 cc	Absence d'hémolyse	Couleur brune verte. Hématine alcaline.
Ch 86	5 cc +	1 cc	Absence d'hémolyse	Zone hémolytique de 3 mm de haut au-dessus du sédiment érythrocytaire. Liquide surnageant inchangé.
Ch 86	5 cc +	1 cc	Absence d'hémolyse	id.
Contrôles:				
Extrait de gélose non-ensemencée	5 cc +	1 cc	Absence d'hémolyse	Absence d'hémolyse.
		1 cc	Absence d'hémolyse	Absence d'hémolyse.

On est frappé, à l'examen du tableau II, que le précipité provenant du milieu ensemencé avec la souche de choléra hémodigestive Ch. 91 transforme le sang de chèvre, après 48 heures, en hématine alcaline. Aucune modification ne se manifeste après 24 heures. Cette souche, étudiée il y a quelques mois, produisait un ferment beaucoup plus actif. Déjà après 24 heures de culture, le sang était transformé en hématine. Le précipité provenant de la Ch. 86 se comporte différemment. L'attaque du sang ne va pas plus loin ici que la formation d'une zone hémodigestive au-dessus du sédiment érythrocytaire. La spectroscopie ne montre ici que la présence d'oxy-hémoglobine. Le même phénomène fut aussi obtenu après 3 jours de culture. La répétition de l'expériment ne changea pas les résultats. Ce ferment protéolytique était donc bien capable de liquéfier la gélatine et de déterminer une légère hémolyse; mais une transformation du sang en combinaison plus simple était impossible.

Ces observations semblent indiquer que nous avons affaire à deux ferments. Différents faits plaident également dans ce sens. Au cours d'un examen sur la variabilité des propriétés d'un certain nombre de souches de choléra, je fus frappé que des cultures, qui liquéfient assez fortement la gélatine, ne présentaient aucune zone hémodigestive sur plaque au sang, tandis que des souches, qui attaquaient la gélatine beaucoup moins intensément, formaient sur la plaque au sang une jolie zone verte et claire. Si l' autodigestion et l'attaque de la gélatine étaient des manifestations d'un seul ferment, une zone aurait dû se former sous l'action d'une souche fortement liquéfiante, et la présence d'une zone hémodigestive dépendrait de la formation de ferment en quantité suffisante. Inversément on rencontre des souches qui possèdent un pouvoir hémodigestif assez grand, mais une propriété liquéfiante beaucoup moins prononcée.

Si les propriétés biochimiques se modifient et si une souche non hémodigestive le devient, le pouvoir de scinder la gélatine semble aussi s'être accru. Tout cela rend vraisemblable que nous avons affaire ici à deux ferments séparés, un ferment hémodigestif et un ferment liquéfiant la gélatine. L'enzyme hémodigestive montre en général une tendance à diminuer rapidement d'activité et à disparaître par culture répétée sur milieux artificiels. Le pouvoir de scinder la gélatine demeure la plupart du temps intact; il peut aussi disparaître, mais le plus souvent diminue seulement d'intensité.

J'ai examiné simultanément le pouvoir hémodigestif et celui de scinder la gélatine de 4 souches de *V. El Tor* repiquées sur gélose et conservées sur un même milieu à l'oeuf composé par Doorenbos

(Alexandrie) *). Les cultures de *V. El Tor* avaient été isolées lors d'une petite épidémie de choléra à Celebes, décrite par DE MOOR (6).

Les propriétés d'attaque de la gélatine furent examinées au moyen du viscosimètre de Ostwald. La technique de cette recherche a été détaillée dans ma thèse. Nous mentionnerons succinctement ce qui suit.

Trois anses normales de chaque souche de cultures de *V. El Tor* âgées de 24 heures, furent déchargées dans un milieu dont voici la composition: 1 % de gélatine (gélatine bactériologique de la „Delftsche Lijm en Gelatine Fabriek" à Delft), 1 % de peptone (Witte), 0,5 % NaCl, eau distillée. Après adaptation du pH à 8.0, addition de 10 % de phosphates-tampon. Des flacons renfermant 70 cc de cette solution de gélatine furent chauffés 3 jours de suite pendant 60 minutes à 70° C. et placés durant 24 heures à 37° C. pour contrôler la stérilité.

L'ensemencement des souches à examiner ayant été fait, la viscosité relative des différents liquides fut determinée après 24, 48 et 72 heures de culture à 37° C. Les différents résultats sont exprimés en courbes (fig. 1 et 2).

Si on compare celles des souches repiquées sur gélose avec celles des cultures conservées sur milieu de DOORENBOS, on gagne l'impression que l'intensité du pouvoir de scission de la gélatine des souches dernièrement nommées a diminué. Vraisemblablement, moins de gélatine est attaquée pendant le même temps. Surtout *V. El Tor* 757, repiqué sur gélose et conservé sur milieu à l'oeuf de DOORENBOS, accuse nettement cette différence. Les autres souches aussi font présumer une réduction.

Le pouvoir hémodigestif des souches repiquées sur gélose se maintient également mieux que celui des cultures sur milieu de DOORENBOS. On peut lire les résultats dans le tableau III.

On voit que chez deux souches, après repiquages sur gélose, l'hémodigestion persiste et croît même un peu, tandis que cette propriété disparaît chez une souche.

Après culture sur milieu à l'oeuf de DOORENBOS, les trois souches

*) Le milieu à l'oeuf de DOORENBOS se prépare comme suit: A. Blanc d'oeuf 10 % dans l'eau distillée; B. Jaune d'oeuf 10 % dans l'eau distillée. A 100 cc de jaune d'oeuf à 10 %, ajouter 1 cc de NaOH normal. A. et B. sont alors chauffés séparément pendant 20 minutes à 100° C. et filtrés sur papier. Mélanger ensuite 750 cc A. et 250 cc B. Répartir en tubes et stériliser à 110° C. pendant 20 à 30 minutes. Ce milieu peut être utilisé pour la longue conservation des bactéries.

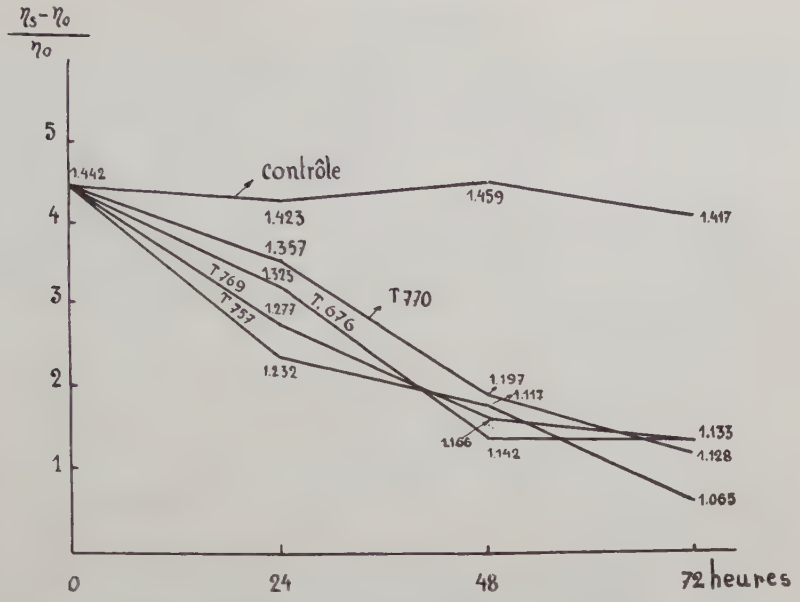

Fig. 1. Souches conservées pendant 4 mois sur le milieu à l'oeuf de Doorenbos (contrôle: solution de peptone gélatinée à 1 %).

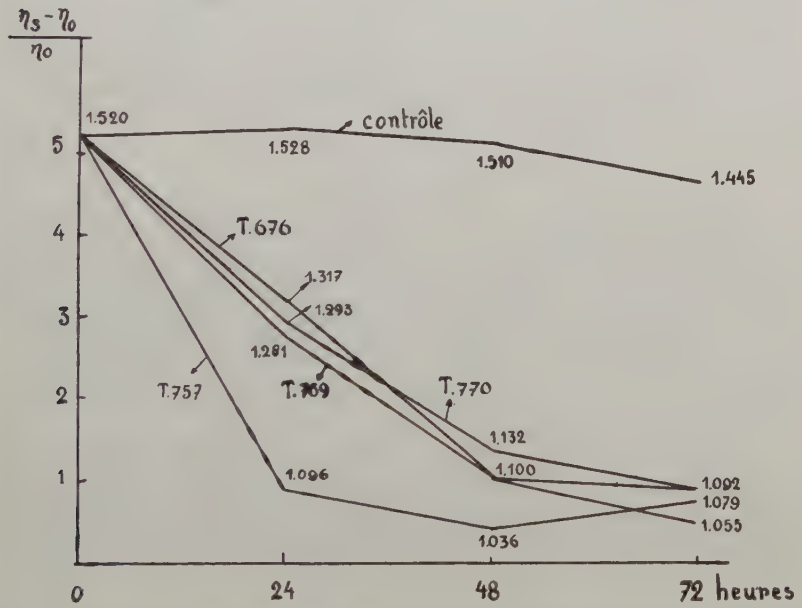

Fig. 2. Souches cultivées pendant 4 mois sur gélose nutritive (contrôle: solution de peptone gélatinée à 1 %).

TABLEAU III.

PROPRIÉTÉS DES SOUCHES DE *V. EL TOR* SUR PLAQUES AU SANG APRÈS REPIQUAGES SUR GÉLOSE ET APRÈS MAINTIEN SUR MILIEU À L'OEUF DE DOORENBOS *).

Souches	Au début Après 1, 2, 3 jours	Après 4 mois de repiquages sur bouillon gélosé. Après 1, 2, 3 jours.	Après un séjour de 4 mois sur milieu de DOORENBOS. Après 1, 2, 3 jours.
V. El Tor 757	c.r. c.v. c.v.	c.v. c.v. c.v.	c.r. c.r. c.r.
V. El Tor 769	c.r. c.r. c.v.	c.v. c.v. c.v.	c.r. c.r. c.r.
V. El Tor 676	c.r. c.v. c.v.	c.r. c.r. c.r.	c.r. c.r. c.r.
V. El Tor 770	c.r. c.r. c.r.	c.r. c.r. c.r.	c.r. c.r. c.r.

*) c.r. = cercle rouge, opaque; c.v. = cercle vert.

ne montrèrent aussi après 3 jours qu'un cercle hémolytique sur plaque au sang.

Ainsi, tandis que le pouvoir hémodigestif des souches de *V. El Tor*, conservées sur milieu à l'oeuf de DOORENBOS, diminue et disparaît, il se manifeste parallèlement une diminution d'attaque de la gélatine par comparaison avec les souches de *V. El Tor* repiquées sur gélose. Deux des 3 cultures repiquées sur gélose nutritive conservent le pouvoir de former une zone verte. Ces observations donnent l'impression que le repiquage régulier sur gélose exerce une influence plus favorable sur le pouvoir hémodigestif qu'une longue conservation sur un milieu qui renferme du blanc d'oeuf.

Conclusions.

On peut conclure, en résumé, que l'hémodigestion et la liquéfaction de la gélatine sont des manifestations de 2 ferments.

Au surplus, les comportements du pouvoir de scission de la gélatine et du pouvoir hémodigestif furent comparés chez 4 souches de *V. El Tor* cultivées sur gélose et conservées sur un milieu renfermant du blanc d'oeuf. A cette occasion on a eu recours au viscosimètre de Ostwald.

Il fut observé que, chez les souches de *V. El Tor* cultivées sur gélose, la scission de la gélatine et l'hémodigestion furent plus intenses que chez des souches conservées sur milieu à l'oeuf. En outre, chez ces dernières, le pouvoir hémodigestif avait entièrement disparu.

Littérature.

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(From the Microbiological Laboratory, Agricultural University, Wageningen.)

DETERMINATION OF THE FERTILITY OF THE SOIL BY MICROBIOLOGICAL METHODS*).

by

K. T. WIERINGA

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The fertility of the soil must be considered as a very complex property. In its most simple conception it is understood as the faculty of the soil to produce a crop, the most fertile soils being those, that produce the largest crop at the smallest expense of money and labour. Among the factors, determining fertility, there appear complex properties, such as structure and absorption capacity. The structure, determining the interrelation of the three phases: solid, liquid, gaseous, is in itself dependent upon the soil colloids, anorganic as well as organic, which in their turn are decisive for the absorptive capacity of the soil. So the presence and availability of the elements necessary for plant-growth must be considered from the same central point of view, the colloidal soil-complex. Of these the cations are present chiefly in an absorbed status. Since, at present, there is an increasing tendency to apply the elements K, P and N in a purified or synthetic form, a growing interest is observed for the so called minor elements **): Fe, Mn, Cu, Zn, B, Mo, e.a. But also Mg and S and the hydrion-concentration, influenced largely by the presence or absence of lime, are important factors.

Apart from these factors the microflora and -fauna claim our special interest. When the question is put, whether a microflora is necessary for plants to produce a crop, the answer will be in the negative. Sterile cultures may be as productive as cultures in a normal fertile soil. In our natural soils, however, the microflora and -fauna cannot be dispensed with. Without the mineralisation process the plant nutrients, present in the organic matter, would

*) Presented at the meeting of the Netherlands Society of Microbiology, Wageningen, May 13th, 1939.

**) Recently the use of the indication "oligo-plerontic elements" has been proposed, *i.e.*, elements required in small quantities (Chronica Botanica Jan. 1940).

be blockaded, and the production of carbon dioxide, being so important in more than one respect, would nearly be brought to a standstill.

On the other hand it is a fact, that the microbiological equilibrium, that establishes itself under the influence of the other factors of soil fertility - the climate of the soil, as pointed out by REMY (21, 22) - modifies itself quantitatively with a change of any of these factors.

All researches for the purpose of studying the microflora and -fauna qualitatively and quantitatively must be considered as attempts to determine the general state of fertility. But there is another group of methods to be considered, intending to determine a certain condition or a certain deficiency, and here micro-organisms may be used as indicators.

When I now enter into discussion on the microbiological methods used for the determination of the general state of soil fertility, I renounce completeness, but I will restrict myself to some general remarks concerning the more important methods, as far as my own experience goes. My own data mentioned in this paper have been collected in studying soil samples partly from the experimental garden of the Microbiological Laboratory in Wageningen, partly from fields in the neighbourhood, or from experimental fields in other regions of the Netherlands.

Beginning with the quantitative determination of micro-organisms in the soil, the well-tried plate method should be mentioned first of all. Though we need not dwell at length upon the errors inherent to this method, attention should be called to a few points. Is is known, that there is no solid medium, that will allow the development of all kinds of micro-organisms, so that only a small part of the total number may be found on the plates. Other imperfections of the method are caused by the discontinuity of the distribution of microbes in the soil (9). The bacteria are found in small colonies on the solid particles, from which they will not easily be seperated. It follows that the figures thus obtained will be too low.

It was known as early as 1909 from a publication by ENGBERDING (7), that each sample of 1 cc of the final dilution gives another outcome, so that it is necessary to use parallels; WAKSMAN (31) uses 8 to 10 plates per sample. As the same mistake is made in the use of every dilution, the same number of parallels should

be used throughout. But then the method would be impracticable. So when it is known in respect of a soil which dilution is most likely to give a countable number of colonies on the plate, it is desirable to prepare this dilution with the least possible stages.

ENGBERDING thinks that notwithstanding these imperfections the plate method is reliable, as the differences between soil samples are of far greater importance than the errors inherent to the method. This only holds true, however, in the case of widely different soil samples. Thorough treatments of the soil and strongly affecting climatic influences can be traced by this method, as was shown by HILTNER and STÖRMER (11) in experiments on partial sterilisation with CS_2.

In the course of my own experiments (in co-operation with J. TEPPEMA) I counted bacteria and actinomyces colonies on soil-extract agar, to which 0.5 % of Ca-caseinate and 0.1 % K_2HPO_4 were added. The soil samples were collected in the neighbourhood of Wageningen, and were partly taken from plots in our garden. Among 23 samples there were clay as well as sandy soils and grassland as well as arable soils. The soil samples were all taken at a depth of 5—10 cm. As soon as the sample reached the laboratory, suspensions were made containing 0.01 mg soil per 0.5 cc, this quantity of the suspensions being plated on the surface of the agar plates in 5 parallels. It is important to dry the plates beforehand on a hot water stove not exceeding a temperature of 70° C. The surface of the plates should be just clearly wrinkled. The agar then having reached a temperature of about 35-40° C., the water will be taken up rapidly and bacterial colonies will not easily spread on the surface. On these plates bacteria and actinomyces grow readily and can easily be recognised, so that after 4 to 5 days the ratio actinomyces: bacteria (a/b) can be determined without the slightest difficulty under these conditions.

Now, as fig. 1 shows, in grassland the ratio $a : b$ is > 1, on arable soil it is just the reverse, while ploughed up grassland after a few years conforms with the arable soil type. So the line $a : b = 1$ marks a division between grassland and arable soil. But a second division may be made, separating clay soils from sandy soils. The total numbers of colonies found in the former are considerably higher than those from the latter types of soil. This is demonstrated by the second line of demarcation in the graph. The field is thus

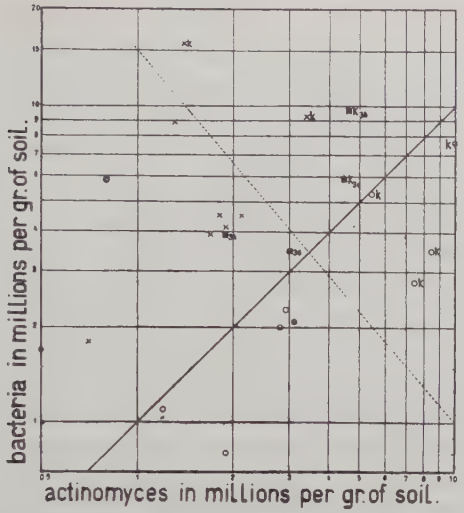

Fig. 1. K = clay; O = grassland; X = arable land; ⊠34 = old grassland ploughed up in 1934 etc. From experiments made during 1936

separated into 4 parts as demonstrated in this diagram:

Arable sand	low numbers	a < b
Grassland sand	low numbers	a > b
Arable clay	high numbers	a < b
Grassland clay	high numbers	a > b.

So it is clear, that large differences in tillage and soil type can be determined by the plate method. Meanwhile it must be stated, that, no matter how useful the data thus obtained, it does not show the total microflora of the soil. The evidence of this omission was emphasized when Conn (6) and later Winogradsky (32) took to directly counting the soil micro-organisms, which was only possible by introducing acid stains (rose bengal, erythrosine) as a means of making the microbes distinguishable between the soil particles. The numbers of bacteria counted with the direct microscopic method many times surpass those obtained by the plate method. Unfortunately the different kinds of bacteria cannot be estimated, nor are we sure whether the bacteria counted were alive or dead in the soil.

The very important observation of Winogradsky's (32), that the majority of the bacteria in the soil are found as colonies, clinging

to the solid soil particles, is a reason the more, for stating that the figures obtained by the plate method are too low. Only where substances (especially organic ones) are added to the soil (culture spontanée) a flora of single cells develops, mostly quite different from the normal autochtonous soil flora. This latter, consisting chiefly of coccus forms, is always present in the soil and is held responsible for the slow decomposition of the organic matter in the soil, whereas the former (mostly rods, with or without spores), has little or nothing to do with the soil microflora proper, coming into the soil as contamination with decaying organic matter. This „wild" flora, however, is the one best studied by soil microbiologists. It develops in the selective nutrient solutions as used in the classical methods of microbiology, e.g., in determining physiological groups numerically by the dilution method (11), or according to their activities by the method of REMY-LÖHNIS (21, 22, 14). These classical methods of microbiology are rejectable, while the microbes are taken away from their normal surroundings, and are placed under abnormal conditions, so that certain species develop that have but little or nothing in common with the autochtonic flora of the soil.

Though this rejecting sentence of WINOGRADSKY's may be justifiable in many respects, it goes too far in other ways, considering the many useful data on the microbiology of the soil, which we have gathered by these classical methods. In fact, all of our knowledge on the processes taking place in the soil is based upon these methods and not in the last place owing to WINOGRADSKY's work. Without any doubt these older methods have yielded practical results of no small importance. I need only refer to the work in our country in the Andyker Proefpolder and Wieringermeer (10). It may be true that the numbers obtained in counting bacteria hydrolysing cellulose, starch, urea, for nitrifiers and denitrifiers, N-fixing organisms etc. are not precisely in accordance with those to be found in the soil, but in comparative researches it is quite possible to distinguish between normal and abnormal soils.

The liquid culture method of REMY and LÖHNIS (l.c.) must be considered less satisfactory. It measures the amount of substrate hydrolysed (starch, cellulose, urea, etc.), oxidized (NH_3) or fixed (N_2) after 2, 4, 6 or 8 days, and from these amounts concludes to the activity of the respective physiological groups of micro-organisms. This method would be valuable if the initial rate of the process could be determined. However this is so low, that it cannot be

measured. But in extreme cases differences in soil types and treatment may be easily demonstrated.

Quite another way was followed by those workers who measured the production of CO_2 in the soil, with or without addition of oxidizable carbon compounds. In relation to these researches the names of HESSELINK VAN SUCHTELEN (29), STOKLASA c.s. (28), PETERSEN (19) and LUNDEGARDH (15) must be mentioned. These workers intended to determine a property of the soil resulting from the co-operation of nearly all organisms living in it, using the soil as a natural medium. Moreover the methods are interesting in so far as CO_2 is a substance in more than one point important for plant nutrition. Without dwelling upon the different modifications of the methods applied in this respect, I would emphasize certain points, that must be considered in estimating the results obtained: 1) Mixing and sieving of the soil will alter its CO_2 production, as was clearly shown by HESSELINK VAN SUCHTELEN (29) Sieved soil gave 177 % more CO_2 than the same soil in unsieved condition. 2) In field experiments, where the soil is left in its natural condition (as far as this is possible) not only the technique of taking a representative sample is very difficult, but it is moreover impossible to eliminate the influence of temperature and moisture on CO_2 production. 3) Where C-compounds are added to the soil sample, only the initial rate of CO_2 production is important. This, however, is so small, that it cannot be determined.

In order to get a better insight into the distribution of microbes and their density, ROSSI (23, 24) and CHOLODNY (3) independantly worked out the soil slide-method. Microscopic slides are buried into the soil and dug up after several days or weeks. After staining with erythrosine, microscopical examination shows what has grown on the glass surface. The local condition of the soil microflora can, according to the authors, thus be studied under natural conditions. KAMERMAN (13), however, showed that the kind of glass used greatly influenced the composition of the microflora, notwithstanding a careful cleaning of the glass.

An extension of this method is that used by ZIEMIECKA (35). Now that it has been proved that the surface of the glass is a factor to be reckoned with, it is an easy step onward to cover it by organic or inorganic substances, thus creating a surface influencing the microflora in a specific way. The organisms growing on the slides will be at the same time dependent on the soil type. I myself made experiments, which showed marked differences in the way of mine-

ralisation of organic matter in different soils. The slides were covered with solutions of starch or peptone and, after drying, were buried in neutral or acid soils. In neutral soils mineralisation does not only progress with greater rapidity, but the microflora developed differs from that grown on acid soils. As the microflora on each slide differs from place to place it is necessary to observe a number of microscopic fields e.g., 25. The results of such an experiment with peptone in a acid soil (pH=5) and a neutral soil (pH=6.8) are found in Table I.

TABLE I.

NUMBER OF FIELDS SHOWING GROWTH OF BACTERIA, FUNGI AND ACTINOMYCES ON PEPTONE-COVERED SLIDES IN 25 MICROSCOPIC FIELDS.

Time in hours	Acid soil			Neutral soil		
	Bact.	Fungi	Actinom.	Bact.	Fungi	Actinom.
8.5	2	0	0	15	0	0
16	10	1	0	22	6	5
21	6	22	0	24	14	6
24	14	21	0	13	16	1
29	15	25	0	16	24	4
40	14	21	0	25	15	18
48	21	19	1	20	16	4
72	17	19	1	18	2	5
96	19	5	3	24	1	9

Differences in neutral and acid soils are also obtained with starch.

Before concluding my discussion of the methods in studying the qualitative and quantitative microflora of the soil I would mention the „soil-chamber" method of Cholodny (4). A square part of a slide of the dimensions of a coverglass is covered with soil, 1 mm high, leaving open a central circular spot of 3 mm in diameter. This square is covered with a coverglass and now placed in a moist chamber. Protruding from the soil into the central room there grows a microflora, that will differ according to the soil. Of course the glass surface must be carefully cleaned. Existing literature gives but little information about results obtained with this method. I myself have observed a development of enormous masses of bacteria of different kinds, rods as well as cocci, in the watery zone, adjoining and surrounding the soil particles. From this I conclude that bacterial numbers in moist soil samples, brought to the laboratory, may alter

greatly in one or two days.

Now let us discuss those methods that intend to determine a certain condition of the soil or a certain deficiency in particular:

In his microscopic study of the soil-organisms WINOGRADSKY (32) regularly observed rather large cocci, resembling *Azotobacter*. To make sure about the true nature of these bacteria it was necessary to bring them to multiplication. For this purpose WINOGRADSKY in the first place used the spontaneous culture ("culture spontanée"). After addition of an N-free C-compound, e.g., mannitol, these cocci multiplied rapidly, and when afterwards this soil was sown on silicogel-mannitol plates, a great number of *Azotobacter* colonies developed. But also without a previous spontaneous culture a number of soils will give rise to a development of *Azotobacter* colonies on silicogel-mannitol plates. According to WINOGRADSKY the occurrence of *Azotobacter* in soils is a characteristic of primary importance for the activity of the soil, those soils not containing *Azotobacter* being inactive. A further step in this line of thought was the development of the method of the "plaque de terre moulée", the soil plaque method. To 50 g of soil 2.5 g of starch or 5 g of mannitol are added, and so much water that a plastic mass is obtained. This mixture is put into a Petri-dish and the surface is smoothed. After 3 days active soils in a moist chamber at 30° C. will show a large number of colonies. Very often these colonies may prove to consist of almost pure cultures of *Azotobacter*. Inactive soils will not give rise to a development of these colonies. According to WINOGRADSKY this method, in combination with the silicogel plate, is the best method for demonstrating the presence of *Azotobacter* in soils.

With inactive soils the soil plaque method often fails to give *Azotobacter*-growth for different reasons. These are: 1) The absence of *Azotobacter*, 2) the missing of a mineral factor (deficiency of P), 3) the presence of an interfering factor (acidity). In many soils lack of chalk is such a limiting factor. *Azotobacter* has been used on a large scale for determining a lack of chalk in an earlier period, when soil scientists could not dispose of our modern methods for the determination of soil acidity, want of chalk and acidity being generally synonymous. It was known, that *Azotobacter* does not develop well in slightly acid media, and in spite of other statements it may be accepted, that *Azotobacter* does not occur in soils with a pH lower than 6.5 and even that it has no practical significance when the pH $<$ 7. Table II taken from a publication of 1926 by WINOGRADSKY (32) shows this.

TABLE II.
(After WINOGRADSKY (32)).

pH de la terre	jours d'étuve	nature des colonies développées sur les plaques.	gain net en azote en milligrammes
		Numération des colonies Azotobacters:	
7,2	10	400	18,53
7,2	10	440	23,33
7,2	10	360 sur toutes les plaques $^1/_2$ à $^1/_2$	20,76
7,2	10	380 est couvert de bulles.	24,59
7,3	10	238	19,75
7,3	10	236	27.64
7,3	10	380	18,87
7,3	7	Azotobacter, 200 colonies. Petit îlot	17,83
7,3	7	de bulles.	17,83
7,3	7		17,91
7,3	7	Azotobacter, 150 colonies. Pas de bulles.	16,87
7,3	7		17,56
6,6	7	Aucune colonie Azotobacter.	0,47
7,4	7	Azotobacter, environ 200 colonies.	18,25
7,0	7	5 colonies Azotobacters.	14,64
7,0	7	Colonies des Azophiles (Bacille gommeux).	0,27
7,0	14	3 colonies Azotobacters qui forment trois grandes flaques, dont deux avec bulles.	7,58
7,1	8		0,57
7,1	8		0,57
7,1	10	Néant d'Azotobacter. Quelques dizaines	1.14
7,1	10	de petites colonies du bacille gommeux.	0,51
7,1	21		0,64
6,6	14	Néant d'Azotobacter. Quelques petites	0,69
6,6	14	colonies d'oligazophiles.	0,72
6,2	12		0,72
6,2	14	Petites colonies d'un coccobacille.	0,85
6,2	14		non dosé
5,9	12		0,40
5,9	14	même remarque.	0,34
5,9	20		non dosé
5,8	12	même remarque.	0,54
5,8	20		non dosé

In Denmark CHRISTENSEN (5) used *Azotobacter* cultures in the following way for determining a lack of chalk. To 50 cc of mannitol-K_2HPO_4 solution 5 grams of soil are added. When this mixture is inoculated with a vigorous *Azotobacter* culture, growth of this organism will only take place when the soil is sufficiently basic. By way of control enough $CaCO_3$ is added to a second culture to warrant basic conditions. In all cases where no film is formed without addition of $CaCO_3$, the soil is in need of chalk.

Now liquid cultures are not favourable to *Azotobacter* growth, and for that reason SÖHNGEN c.s. (27) have substituted a solid medium for CHRISTENSEN's culture medium, adding 1.5 % agar. Furthermore the quantity of chalk necessary for the growth of the *Azotobacter* was determined by adding measured quantities of $CaCO_3$. Further, to promote the reaction between the acid humous substances and the $CaCO_3$ it was necessary to steam the soil in watery suspension with the chalk, the water being poured off after this treatment. Then the soil was mixed with the mannitol-phosphate agar medium and plates were poured in 9 cm Petri dishes. On the surface of these plates *Azotobacter* will readily grow when sufficient chalk is added to reach a pH = 6.8.

It was shown, that there was a linear relation between want of bases and the humus content, this want of bases being more or less satisfied in normal soils. It was shown besides that our normal Dutch sandy soils have an acid reaction, whereas our fertile clay soils are alcaline or at least neutral. Therefore the often accepted opinion that *Azotobacter* must be present in fertile soils does not hold with regard to humous sandy soils. Fig. 2. shows a series of mannitol-agar-soil plates from differently manured experimental plots in the garden of the Laboratory of Microbiology at Wageningen. The manuring of these soils is given in Table III. Where *Azotobacter* grows on the plates, films of this organism will usually develop in liquid cultures without extra inoculation of the mannitol-phosphate medium, and also in WINOGRADSKY's soil plaques *Azotobacter* colonies will develop, provided that other necessary mineral factors are present.

In America WINOGRADSKY's soil plaque method (see also (33) and (34)) has been supplemented by the inoculation principle, already used by CHRISTENSEN in his liquid cultures. By adding $CaCO_3$ at the same time, one may be sure that the inoculated *Azotobacter* will grow when other mineral factors are present in sufficient quantities.

TABLE III.

PLAN OF TREATMENT OF THE EXPERIMENTAL FIELD OF THE LABORATORY OF MICROBIOLOGY AT WAGENINGEN.

1) Sand	9) Sand + Peat-litter	17) Complete manuring. l.s. = -16	25) No P l.s. = -10	33) Clay
2) Sand + 2% lime-marl	10) Sand + Peat-litter 2% lime-marl	18) No P l.s. = -11	26) Complete manuring. l.s. = -17	34) Clay
3) 60 kg. lime-marl l.s. = 0 †)	11) 5 kg. lime-marl l.s. = -16	19) 15 kg. lime-marl l.s = -11	27) No manuring. l.s. = -10	35) Complete N as $NaNO_3$ l.s. = -13
4) 30 kg lime-marl l.s. = -4	12) 0 kg. lime-marl l.s. = -20	20) 10 kg. lime-marl l.s. = -14	28) No N l.s. = -14	36) Complete N as sulf. of ammonia l.s. = -25
5) 15 kg. lime-marl l.s. = -12	13) 60 kg. lime marl l.s. = 0	21) 5 kg. lime-marl l.s. = -20	29) No K l.s. = -15	37) Farmyard manuring. l.s. = -7
6) 10 kg. lime-marl l.s. = -15	14) 30 kg. lime-marl l.s. = +2	22) 0 kg. lime marl l.s. = -21	30) Complete N as $NaNO_3$ l.s. = -14	38) No manuring. l.s. = -11
7) 5 kg. lime-marl l.s. = -16	15) 15 kg. lime-marl l.s. = -11	23) 60 kg. lime-marl l.s. = 0	31) Complete N as sulf. of ammonia. l.s. = -24	39) No N l.s. = -13
8) 0 kg. lime-marl l.s. = -20	16) 10 kg. lime-marl l.s. = -14	24) 30 kg. lime-marl l.s. = -4	32) Farmyard manuring. l.s. = -10	40) No K l.s. = -12

†) l.s. = lime status. Cf. HUDIG (12).

Fig. 2. Soil samples in mannitol-agar soil plates, inoculated with a pure culture of *Azotobacter*. Experimental field plots from the Laboratory of Microbiology at Wageningen.

In this form SACKETT c.s. (25, 26) have used the soil plaque method for the determination of P and K deficiency of the soil. The classification of soils in types with respect to phosphate deficiency is shown in Fig. 3.

In Denmark PETERSEN (20) tested SACKETT s method, comparing his results with those of experimental plots and observations in the field. He stated, that *Azotobacter* will strongly react upon deficiency of P, perhaps a little more strongly than plants (Cf. Fig. 4). The reaction upon a lack of K was less satisfactory. This is quite in accordance with the statements of GERLACH and VOGEL (8) and VOGEL (30). These authors even go so far as to assert that *Azotobacter* does not need any K at all for its growth.

My own observations indicated, that it will be necessary to add other mineral elements to the substrate, when deficiency of K or P must be determined. In leached out acid soils addition of Mg and Mo will often be necessary. In 1935 already VAN NIEL (17) drew attention to the importance of this means of determining the absence of

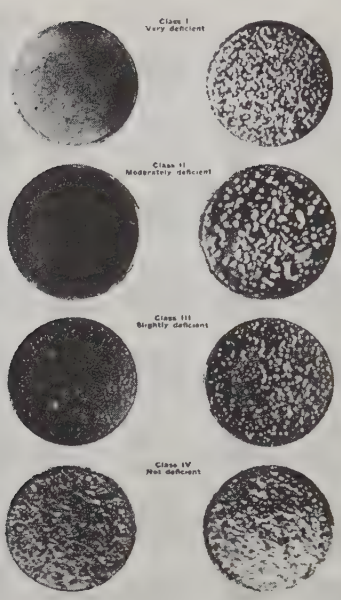

Fig. 3. Type plaques for phosphate deficiency classification. Left row: Checks, nothing added; right row: phosphate added (After Stewart. Sackett Robertson and Kezer (26)).

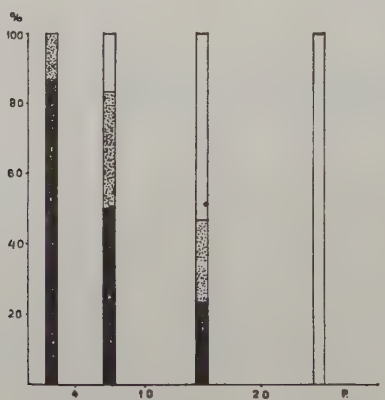

Fig. 4. Percentage of soil samples deficient in P. at different P. Levels.
■ P. deficiency.
▨ Intermediate Cases.
□ No P. deficiency

Mo in soils, and in 1937 Bortels (1) used the same method. But also deficiency of Mg, often found in acid sandy soils can easily be ascertained by the soil plaque method.

As was already shown by Petersen (20), the reaction of *Azotobacter* in regard to P deficiency is stronger than that of plants. I can completely confirm this statement. In our experimental garden all plots except clay-soils need addition of phosphate to the soil plaques, whereas vegetation is not appreciably stimulated by phosphate manuring. It is possible that this difference in reaction of *Azotobacter* and plant is caused by the artificial basicity, following the addition of $CaCO_3$ to the soil plaques, which makes the phosphate ion less soluble.

An acid reaction does not interfere with the *Cunninghamella* method of P estimation. Here the fungus *Cunninghamella* is used instead of *Azotobacter;* nitrogen is added to the culture medium as NH_4NO_3 and peptone, and the pH is kept below 7.5. After 48 to 50 hours at 28-29° C, the diameter of the fungus colony is taken as a measure for the amount of P present. (See Fig. 5). Mehlig Fred and Truog (16) got very good results with this method, though these could not be confirmed by others.

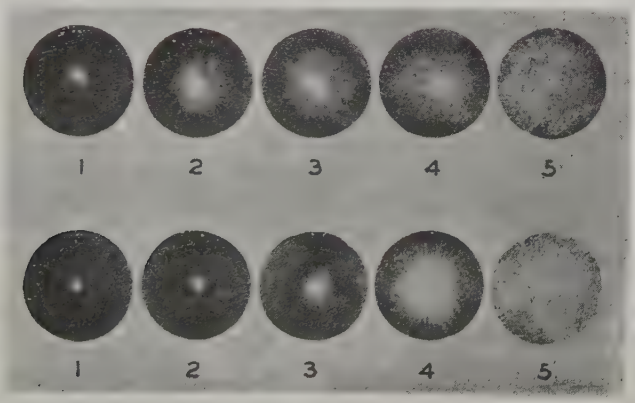

Fig. 5. Growth of *Cunninghamella* on soils with different amounts of available phosphorus. Upper row: a soil treated with increasing amounts of soluble phosphorus. Lower row: soils untreated, containing increasing amounts of phosphorus (After Mehlig, Fred and Truog (16)).

In conclusion the *Aspergillus* method should be mentioned, largely used by Niklas c.s. (18) to establish a lack of P and potash, and used by other investigators to show a deficiency of copper and

magnesium in soils. This subject will amply be dealt with in publications by the hand of Dr F. C Gerretsen and of Dr. E. G. Mulder.

Summary.

A review is given of the determination of soil fertility by microbiological methods. The quantitative and the qualitative methods for the determination of the microflora were discussed as well as those methods in which micro-organisms are used for ascertaining a particular condition or a limiting factor in the soil. More especially the use of *Azotobacter* cultures for this purpose was mentioned.

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(Aus der mikrobiologischen Abteilung an der Rijkslandbouw proefstation, Groningen.)

PHOSPHAT-BESTIMMUNGEN MITTELS *ASPERGILLUS NIGER*

von

F. C. GERRETSEN und NORA BLUMENDAL.

(Eingegangen am 27 Oktober 1939)

1. EINLEITUNG.

Die Bestimmung des Phosphatbedürfnisses von Ackerböden mittels des Schimmelpilzes *Aspergillus niger*, welche von NIKLAS und seinen Mitarbeitern (12) ausgearbeitet wurde, bietet noch immer gewisse Schwierigkeiten, welche der praktischen Anwendung dieser Methode im Wege stehen [1]).

Ihrer Einfachkeit und Billigkeit wegen würde aber die Methode gerade zu diesem Zweck besonders geeignet sein. Deswegen haben wir versucht die Bedingungen für eine exakte Bestimmung näher zu präzisieren und die Methodik soweit wie möglich damit in Übereinstimmung zu bringen.

Der Ertrag eines lebenden Organismus ist eine komplizierte Grösse, welche nicht in einfacher Weise von einem Faktor, welcher im Minimum anwesend ist, abhängt.

PRINGSHEIM (15) hat schon im Jahre 1914 an der Hand von Versuchen mit *Aspergillus niger* darauf hingewiesen, dass die Ausnützung eines Nährstoffes, der im Minimum vorhanden ist, und damit die Produktionssteigerung, von der Menge der anderen Nährstoffe abhängig ist.

Die mathematische Formulierung des Zusammenhanges zwischen

[1]) Bei dieser Methode wird zu einer bestimmten Menge Nährflüssigkeit, welche alle nötigen Nährstoffe enthält, ausser Phosphorverbindungen, eine abgewogene Menge Boden hinzugefügt; der Pilz entwickelt sich im Verhältniss zu den im Boden anwesenden assimilierbaren Phosphorverbindungen. Man bestimmt nach vier Tagen das Gewicht des Pilzmyzels.

Ertrag und Wachstumsfaktoren von MITSCHERLICH gibt auch für *Aspergillus niger* kein genaues Bild, wie durch MEYER (7) dargelegt wurde. Es sind wenigstens drei Faktoren, welche einer einfachen mathematischen Formel im Wege stehen: erstens das Anpassungsvermögen der lebenden Organismen an bestimmte ungünstige Verhältnisse, was u.a. die Ursache dafür ist, dass die prozentische Zusammensetzung des Trockenmaterials innerhalb gewisser Grenzen schwanken kann, ohne dass sich dies im Ertrag deutlich widerspiegelt. So geht aus dem Versuch auf Seite 91 deutlich hervor, dass das P_2O_5-Gehalt des Myzels von *Aspergillus niger* bei niederigem Phosphatgehalt der Nährflüssigkeit höher ist als bei mittlerem Gehalt, während es bei den höchsten Konzentrationen wieder zunimmt.

Ein zweiter Faktor ist der Einfluss von Wuchsstoffen und Spuren-Elementen: mit der An- oder Abwesenheit von einigen Gamma dieser Stoffe kann sich der Ertrag sprungweise um mehrere Zehnerprozente ändern. So war von KIESSLING (5) bei der Untersuchung verschiedener Böden angegeben worden, dass durch Hinzufügung kleinerer Mengen Humussäure (0,5%) die Myzelgewichte um 3,3—50,5% heraufgehen können. Bei unseren Versuchen wurden sogar durch Hinzufügung von 0,016% Huminsäure Mehrerträge von 9 bis 40% erhalten.

Drittens hat man darauf zu achten, dass der Pilz in einer beschränkten Menge Nährflüssigkeit wächst; infolge dessen ist die Zusammensetzung des Mediums nicht konstant, sondern ändert sich während des Wachstums ständig. Insbesondere bezieht sich dies auf die Bildung von Säuren und Änderungen des pH's, welche gerade auf die Phosphatbestimmungen von grossem Einfluss sein können.

Es ist selbstverständlich, dass die obengenannten Faktoren eine mathematische Interpretation der Myzelium-Ernten ausserordentlich erschweren, es sei denn, dass es gelingt, die Zusammensetzung der Nährflüssigkeit derart zu gestalten, dass dieselbe, vorbehaltlich des Phosphatgehaltes, dem Schimmelpilz optimale Bedingungen darbietet.

Auch NIKLAS c.s. hatten schon darauf hingewiesen (l.c. HONCAMP's Handbuch, S. 850), dass es unbedingt nötig ist „dass selbst der Zusatz verschiedener Böden, trotz der in ihnen enthaltenen Nähr- und Reizstoffe, auf die Entwicklung und Ernte des Pilzes keinen wesentlichen Einfluss mehr auszuüben vermag".

Bei unseren Untersuchungen zeigte es sich aber, dass die von NIKLAS und Mitarbeitern zusammengestellte Nährflüssigkeit den gestellten Ansprüchen nicht genügt. Von TRISCHLER (18) und auch von VILSMEIER (19) war schon auf die ziemlich grossen pH-Änderungen hinge-

wiesen worden, die während des Pilzwachstums in dem Kulturmedium auftreten. Mit Rücksicht auf die Abhängigkeit der Phosphatlöslichkeit vom pH betrachtete Söding (17) dies sogar als eine ernste Unzulänglichkeit der Methode.

Auch war von Niklas c.s. (13) schon selbst konstatiert worden, dass der Kalkgehalt des Bodens die Myzel-Ernte stark beeinflusst, nicht nur in Folge der Pufferwirkung, sondern auch weil Kalzium für den Pilz ein Nährstoff ist. Deswegen bekommt man mit kalkhaltigen Böden zu hohe Werte, auch wenn das $CaCO_3$ mit Zitronensäure neutralisiert worden ist.

Diese Schwierigkeit wird weder von Niklas, noch in den darauf folgenden Untersuchungen von Vilsmeier (l.c. S. 285) entscheidend gelöst; u.E. wird sogar durch den Vorschlag, auf Grund eines ausgedehnten Untersuchungsmaterials empirische Werte festzustellen, die ganze Methode gelockert.

Es ist also selbstverständlich, dass unter diesen Umständen der Phosphatbestimmung mittels *Aspergillus niger* gewisse Fehler anhaften, welche der Zuverlässigkeit der Methode im Wege stehen.

Es ist unsere Aufgabe gewesen, diese Unzulänglichkeiten so weit wie möglich zu beseitigen, damit bei Anwendung der Methode nicht nur „in der Hand des geschulten Fachmannes noch (zahllose) andere Faktoren unbedingt berücksichtigt werden müssen" (Niklas c.s. (14) l.c. S. 66) sondern sie auch ohne weiteres bei der Bodenuntersuchung von einfach geschulten Laboranten ausgeführt werden kann.

2. METHODIK.

P i l z k u l t u r e n. Die Versuche wurden angestellt mit dem von Niklas und Mitarbeitern gebrauchten *Aspergillus niger*-Stamm (Poschenrieder), der uns in kollegialer Weise zur Verfügung gestellt wurde, und daneben mit einem von uns in üblicher Weise von Korinthen isolierten *Aspergillus niger*-Stamm (Groningen). Dieser zweite unterschied sich von dem ersten dadurch, dass er Harnstoff als Stickstoffquelle gebrauchen konnte, dass er kein Pepton zum üppigen Wachstum benötigte und ein optimales pH von 3,5 hatte statt 2,5, was mit Hinsicht auf die Pufferung der Lösung ein Vorteil ist, während das Wachstum eventueller Infektionen auch bei diesem pH durchaus zu vernachlässigen ist.

Die I m p f u n g der Kulturen geschah mit 0,5 ccm einer 6 Tage alten Sporensuspension; es zeigte sich, dass die Menge des Impfmaterials einen wesentlichen Einfluss auf die Myzel-Ernte ausübte, weshalb

es unbedingt notwendig ist bei vergleichenden Untersuchungen immer mit der gleichen Menge homogenen Sporenmaterials zu impfen.

Die Sporen wurden in Erlenmeyerkolben von 300 ccm mit 75 ccm der üblichen Kulturflüssigkeit gezüchtet, welche zu diesem Zweck nur 0.002% P_2O_5 enthielt. Nach 6 Tagen bei 37° C. wurde das Myzel abgetrennt, wiederholt mit destilliertem Wasser gewaschen, darauf mit 50 ccm sterilem Wasser tüchtig geschüttelt und schliesslich durch ein nichrom Gaze filtriert.

Die Kulturflüssigkeit war anfänglich dieselbe, wie die von NIKLAS c.s. benutzte; sie enthielt:

		%
Saccharose		10
Zitronensäure		1
Pepton		0,1
$(NH_4)_2SO_4$		0,6
P_2O_5	$(NH_4H_2PO_4)$	0,075
K_2O	(K_2SO_4)	0,02
$MgSO_4$		0,03
Cu	$(CuSO_4)$	0,00015
Zn	$(ZnSO_4)$	0,0001
Fe	$(FeSO_4)$	0,0001

Das pH dieses Mediums ist etwa 2,5; zur Kultur des Stammes Groningen wurde das pH durch Hinzufügung von 0,2% NaOH auf 3,5 gebracht.

Um genügend genaue Bestimmungen bekommen zu können, benutzten wir zur Kultur des Pilzes Erlenmeyerkolben von 300 ccm Inhalt, welche mit 75 ccm Nährflüssigkeit beschickt wurden. Die Bestimmungen wurden in triplo oder in quadruplo ausgeführt.

Zur Ernte des Myzeliums wurden pünktlich nach 96 Stunden einige ccm Formalin 40% hinzugefügt, und die Kulturen über Nacht bei Zimmertemperatur stehen gelassen. In dieser Weise werden nicht nur Pilz und Sporen getötet und Infektionen verhütet, sondern das Myzel wird sogleich gegerbt und es lässt sich nachdem leicht behandeln ohne zu zerreissen.

Die Unterseite des Myzels wird zuerst mit Leitungswasser vorsichtig abgespritzt, nachher mit destilliertem Wasser übergossen, auf ein gewogenes Filter gebracht und noch einige Male mit kochendem Wasser gewaschen.

Das Trocknen geschieht in zwei Stadien, zuerst 14—16 Stunden bei 55°C., nachher noch 3 Stunden bei 105°C.

3. DIE ZUSAMMENSETZUNG DES KULTURMEDIUMS.

Der Pepton-Zusatz.

Im allgemeinen wird durch Peptonzusatz die Infektionsgefahr einer Nährflüssigkeit gesteigert; während das pH von 2,5, das in den Kulturen von NIKLAS c.s. besteht, Infektionen auf ein Minimum beschränkt, ist bei dem pH von 3,5, das wir bei unseren Versuchen vorziehen, die Infektionsgefahr bei Anwesenheit einer so leicht angreifbaren Stickstoffquelle nicht zu vernachlässigen. Auch spielt bei Massenuntersuchungen der ziemlich hohe Preis des Peptons eine wesentliche Rolle.

Aus diesen Gründen war es ein Vorteil, dass unser *Aspergillus*-Stamm Groningen sich ohne Pepton normal entwickelt. Er wird sogar durch Hinzufügung von 0,1% Pepton deutlich gehemmt (Tabelle I).

Tabelle I.

Hemmung von *Aspergillus niger* (Groningen) durch 0,1% Pepton

P_2O_5-Gehalt in %	Myzelgewicht in g [1]	
	mit Pepton	ohne Pepton
0,0	0,007	0,009
0,001	0,089	0,143
0,002	0,234	0,303
0,004	0,501	0,644
0,006	0,657	0,840
0,008	0,809	1,018
0,010	0,987	1,338

[1]) Kulturzeit 6 Tage bei 37° C. Mittel von vier Parallelbestimmungen.

Wir sehen also, dass bei allen Konzentrationen das Myzelgewicht ohne Pepton bedeutend höher ist als dasjenige mit Pepton; deshalb wurde bei den Versuchen mit unserem *Aspergillus*-Stamme Groningen das Pepton fortgelassen.

Die Mengen der Spuren-Elemente Fe, Zn, Cu und Mn.

TRISCHLER fügt seiner Nährlösung folgende Mengen Spuren-Elemente hinzu: 0,00015% Cu, 0,0001% Fe und 0,0001% Zn, alles als Sulfate. Es zeigte sich, dass eine Steigerung des Eisengehalts von 0,0001% auf 0,0005% und eine Verdopplung des Zinkgehalts bei einem Gehalt von 0,006% P_2O_5 das Myzelgewicht um 21% vergrösserte.

Auch konnte ohne Schaden die Menge des Kupfers bis auf ein Drittel herabgesetzt werden.

Mangan wurde von TRISCHLER nicht in seiner Kulturflüssigkeit gebraucht; es zeigten sich aber während unserer Untersuchungen mit dem Stamm POSCHENRIEDER bisweilen Wachstumsstörungen: das Myzel war nicht zusammengewachsen und faserig, während die Ernte zu gering war. Diese Erscheinung wurde niemals beobachtet, wenn den Kulturen ein wenig Boden oder Bodenextrakt hinzugefügt worden war. Auch SMITH (16) hatte derartige Schwierigkeiten mit seinen *Aspergillus*-Kulturen beobachtet und schrieb sie dem Mangan-mangel zu.

In der Tat zeigte es sich, dass durch Hinzufügung von 0,0001% Mn als $MnSO_4$ zur Nährlösung TRISCHLER's das Wachstum des *Aspergillus*-Stammes POSCHENRIEDER wieder normal wurde. Die Vermutung, dass der Mn-Gehalt des Rohrzuckers während des Umkristallisierens unter den Grenzwert gesunken war, wurde nicht bestätigt (Tabelle II).

Tabelle II.

Einfluss von Mn auf *Aspergillus niger* (Stamm POSCHENRIEDER).

P_2O_5-Gehalt in %		Myzelgewicht in g [1])	
		Ohne Mn	mit 0,0001% Mn
0,01	Handelszucker	1,24	1,85
0,01	„ umkrist.	1,19	1,81

[1]) Kulturzeit 4 Tage bei 37° C.

4. DIE FAKTOREN, WELCHE DIE pH-ÄNDERUNGEN DES KULTURMEDIUMS BEDINGEN.

Wegen des entscheidenden Einflusses des pH's auf die Löslichkeit der Phosphate ist es unbedingt nötig, dass das pH des Kulturmediums am Anfang und am Ende des Versuchs so weit wie möglich gleich bleibt.

Von den Faktoren, welche die pH-Änderungen bedingen, nennen wir:

I. Die Stickstoffquelle, i.c. Ammoniumsulfat; je besser der Pilz wächst und je mehr Ammoniumstickstoff er assimiliert, um so mehr H_2SO_4 bleibt zurück. Dies ist allerdings die wichtigste Ursache des Sauerwerdens des Kulturmediums.

II. Dasselbe trifft zu für die Sulfate von K, Mg und Ca, sei es auch in minderem Masse.

III. Die vom Schimmelpilz aus dem Zucker gebildeten organischen Säuren wie Zitronen-, Oxal- und Gluconsäure.

IV. Die Pufferkapazität des Kulturmediums; diese resultiert aus derjenigen der Kulturflüssigkeit an sich und der des zugefügten Bodens. Die Pufferung der Nährflüssigkeit ist nur gering; diejenige des Bodens ist hauptsächlich abhängig vom Humusgehalt, vom Gehalt an kolloidalen Teilchen und an $CaCO_3$. Weil bei den Versuchen das im Boden vorhandene $CaCO_3$ mit Zitronensäure neutralisiert wird, hat man mit der Pufferwirkung des Kalziumzitrats zu tun. Es ist deutlich, dass es einen grossen Unterschied macht, ob man mit einem humusarmen Sandboden oder mit einem reichlich mit $CaCO_3$ versehenen, schweren Lehmboden zu tun hat. Die Pufferwirkung des Kulturmediums ist also ein recht komplizierter und ungewisser Faktor.

V. Die Menge des im zugefügten Boden vorhandenen Phosphats; je mehr Phosphat dem Pilz zur Verfügung steht, um so besser wächst er und um so stärker kommen die unter I—III genannten Faktoren zum Ausdruck. Im entgegengesetzten Sinne wirkt aber die zunehmende Menge der gebildeten Säure, welche die Myzelbildung hemmt.

Aus den angeführten Gründen wird es deutlich, dass das resultierende Myzelgewicht keinenfalls vom Phosphatgehalt des Bodens allein abhängig ist. Dass das End-pH in engster Beziehung zu der Menge des assimilierten Phosphats steht, geht deutlich aus den folgenden, den Arbeiten von Niklas, Poschenrieder und Trischler entnommenen Zahlen hervor (Tabelle III).

Tabelle III.

nach Niklas, Poschenrieder und Trischler (12).

	End-pH nach 4 Tagen
Nährlösung, ungeimpft	2,5
mit 0,0025% P_2O_5	1,99
mit 0,005 % P_2O_5	1,69
mit 0,0075% P_2O_5	1,45
mit 0,010 % P_2O_5	1,38
mit 0,050 % P_2O_5	1,29

Durch Hinzufügen von Böden steigt überdies das Anfangs-pH

bisweilen von 2,5 bis 3,0—3,5, wodurch das pH-Trajekt noch erweitert wird. Andererseits ist es zu erwarten, dass die Phosphatmengen, welche aus den Böden in Lösung gehen, in hohem Masse vom pH abhängig sind. Dies geht deutlich aus den folgenden chemischen Bestimmungen derselben Untersucher hervor (Tabelle IV).

Tabelle IV.
nach Niklas, Vilsmeier und Kohl (14).

Muster Nr. des Bodens	Boden extrahiert mit 0,25% Zitronensäure; pH 2,54	Boden extrahiert mit 4,0% Zitronensäure; pH 1,92	Mehr in %
25	4,28 mg P_2O_5 in 100 g Boden	6,87 mg P_2O_5 in 100 g Boden	56,7
16	6,77 „ „	11,37 „ „	68,0
7	8,78 „ „	13,27 „ „	51,2
15	21,9 „ „	29,6 „ „	35,2
4	15,2 „ „	71,9 „ „	367,0
12	70,0 „ „	138,4 „ „	97,7

Auch die P_2O_5-Bestimmungen mittels *Aspergillus* bei verschiedenem Anfangs-pH in Böden geben Werte, welche sehr weit auseinander liegen (Tabelle V).

Tabelle V.
nach Niklas, Vilsmeier und Kohl (14).

Boden Nr.	Myzelgewicht in g nach 5 Tagen		
	Anfangs-pH 2,73	Anfangs-pH 2,25	Anfangs-pH 2,03
25	0,678	0,846	1,120
7	0,561	1,105	2,012
15	1,644	2,183	2,726
4	0,901	1,909	3,738
23	2,618	3,082	4,066 [1])

[1]) pH = 1,99.

Trotzdem also aus dem von Niklas c.s. selbst erhaltenen Material deutlich hervorgeht, welchen grossen Einfluss das pH auf die Menge Phosphorsäure hat, die man entweder chemisch oder mikrobiologisch bestimmt, haben diese Untersucher leider dieser Tatsache keine weitere Aufmerksamkeit geschenkt. Sie schreiben darüber: „Um die Methode

nicht unnötig zu komplizieren, lässt man am besten den Anspruch auf gleiche End-pH fallen." (11).

Dies kommt darauf hinaus, dass man den Phosphatgehalt verschiedener Böden bei ganz verschiedenen pH bestimmt; wie aus den Tabellen hervorgeht, können bei einer pH-Differenz von weniger als 1, die Werte um mehr als 300% differieren (Tabelle IV und V, Nr. 4).

Dass man auf diesem Wege vergleichbare Resultate bekommen könnte, ist nicht zu erwarten; dazu wäre es unbedingt nötig, die pH-Änderungen in enge Grenzen zurückzudrängen.

Der Einfluss der Stickstoffquelle auf die pH-Änderungen.

Die wichtigste Anforderung, welche an die Stickstoffquelle, ausser guter Assimilierbarkeit, zu stellen ist, ist die Abwesenheit eines anorganischen Säurerestes. Es ist der SO_4''-Teil des Ammoniumsulfats, der für die pH-Abnahme an erster Stelle verantwortlich ist.

Es lag auf der Hand, das $(NH_4)_2SO_4$ durch NH_4NO_3 zu ersetzen, das im Allgemeinen als eine physiologisch neutrale Verbindung betrachtet wird. Nach den Untersuchungen von SCHLOESING, BUTKEWITSCH, NIKITINSKY u.a. praeferiert aber *Aspergillus niger* den Ammonium-Teil dieses Moleküls und von BRENNER (3) wurde sogar gezeigt, dass das Kulturmedium in Folge des Freiwerdens der Salpetersäure stark sauer wird. Vom zugefügten Ammoniumnitrat wurde fast 90% des darin anwesenden Ammoniakstickstoffs assimiliert, während 80% des Nitratstickstoffs als freie Salpetersäure in der Flüssigkeit nachweisbar war. Diese Verbindung ist also für unserem Zweck unbrauchbar.

Organische Säurereste sind in dieser Hinsicht weniger zu fürchten; hier aber kann die undissozierte Säure eine ausgesprochene Giftwirkung ausüben.

Die ersten Versuche wurden mit *Aspergillus niger*, Stamm POSCHENRIEDER, angestellt; die Nährlösung war dieselbe, die von NIKLAS c.s. benutzt wurde und deren Zusammensetzung auf Seite 74 angegeben wurde. Nur die Stickstoffquelle wurde geändert und durch die folgenden Verbindungen ersetzt in einer Menge im Liter, die dem N-Gehalt einer 0,6%-igen Ammoniumsulfat-Lösung equivalent war:

I. 6 g Ammoniumsulfat
II. Ammoniumzitrat (Lösung)
III. 12 g Ammoniumazetat
IV. 9,5 g Ammoniumlaktat
V. 6 g Asparagin

VI. 3 g Harnstoff
VII. 3 g Ammoniumsulfat + 1,5 g Harnstoff
VIII. Ammoniumzitrat + 1,5 g Harnstoff
IX. 2 g Ammoniumsulfat + 4 g Asparagin
X. Ammoniumzitrat + 4 g Asparagin.

Das Ammoniumzitrat wurde hergestellt, indem 500 ccm 2,15 n Zitronensäure mit 500 ccm 2,15 n Ammoniaklösung neutralisiert wurde; zu 1 Liter Nährflüssigkeit wurde 90 ccm dieser Ammoniumzitratlösung hinzugefügt. Das pH dieser Lösung war 4,5; um einen Vergleich mit der Originalnährlösung zu erleichtern, wurde das pH durch Hinzufügung von 15 g Zitronensäure pro Liter auf etwa 3,2 gebracht. Dasselbe geschah mit Lösung III.

In Tabelle VI sind die Resultate dieser Versuche bei verschiedenen P_2O_5-Konzentrationen zusammengestellt.

Aus den Zahlen der Myzelgewichte ist ersichtlich, dass Ammoniumsulfat und -Zitrat als N-Quelle gleichwertig sind; Azetat und Laktat hingegen sind völlig unbrauchbar. Dies wird offenbar durch die Giftwirkung der undissozierten Säuren verursacht, denn durch Hinzufügung von Essig- oder Milchsäure zu Lösung I wird die Entwicklung des Myzels fast völlig gehemmt.

Asparagin zeigte sich als ziemlich brauchbar, während Harnstoff, besonders bei Anwesenheit von mehr P_2O_5, zurückbleibt. Von den Mischungen VII—X bleiben auch diejenige mit Harnstoff hinter den anderen zurück.

Was die pH-Änderungen anbelangt, sehen wir, dass merkwürdigerweise zwischen Ammoniumsulfat und -Zitrat nur wenig Unterschied besteht; bei 0,01% P_2O_5 betrugen die Änderungen resp. —0,97 und —0,87.

Asparagin und Harnstoff unterscheiden sich von den anderen N-Quellen dadurch, dass das pH nicht abnimmt, sondern steigt, am meisten bei Harnstoff mit sogar + 0,72 pH (0,002% P_2O_5). Dies ist gewiss eine Folge der enzymatischen Spaltung dieser beiden Verbindungen, wobei Ammoniak ins Freie gesetzt wird.

Deswegen sehen wir auch, dass die pH-Änderungen in den Mischungen, die Harnstoff oder Asparagin enthalten, geringer sind als in denjenigen ohne diese Verbindungen.

Obwohl wir also mit diesem *Aspergillus*-Stamm einige wertvolle Hinweise erhielten, war das Wachstum mit der Originalnährlösung Niklas' am besten und keine dieser Lösungen entsprach der Forderung, dass ein maximales Wachstum bei gleichem Anfangs- und

Tabelle VI.

Der Einfluss der Stickstoffquelle auf Myzelertrag und pH (*Aspergillus niger* Stamm POSCHENRIEDER).

N-Quelle	0,002% P_2O_5			0,006% P_2O_5		
	Myz. Gew. in g[1])	Anfangs-pH	End-pH	Myz. Gew. in g[1])	Anfangs-pH	End-pH
I. Ammoniumsulfat	0,31	2,29	1,98	1,01	2,29	1,56
II. Ammoniumzitrat	0,33	3,24	2,81	0,95	3,17	2,58
III. Ammoniumazetat	0,00	3,50	—	0,00	3,50	—
IV. Ammoniumlaktat	0,00	3,50	—	0,00	—	—
V. Asparagin	0,24	2,39	2,51	—	—	—
VI. Harnstoff	0,21	2,19	2,91	—	—	—
VII. Ammoniumsulfat + Harnstoff	0,16	2,41	2,55	0,68	2,43	2,32
VIII. Ammoniumzitrat + Harnstoff	0,15	3,43	3,45	0,74	3,47	3,09
IX. Ammoniumsulfat + Asparagin	0,32	2,55	2,42	0,74	2,53	2,37
X. Ammoniumzitrat + Asparagin	0,30	3,18	2,76	0,84	3,18	2,63

N-Quelle	0,008% P_2O_5			0,010% P_2O_5		
	Myz. Gew. in g[1])	Anfangs-pH	End-pH	Myz. Gew. in g[1])	Anfangs-pH	End-pH
I. Ammoniumsulfat	—	—	—	1,37	2,29	1,32
II. Ammoniumzitrat	—	—	—	1,45	3,17	2,30
III. Ammoniumazetat	0,00	3,50	—	—	—	—
IV. Ammoniumlaktat	0,00	3,50	—	—	—	—
V. Asparagin	1,13	2,39	2,79	—	—	—
VI. Harnstoff	0,86	2,19	2,81	—	—	—
VII. Ammoniumsulfat + Harnstoff	—	—	—	1,10	2,43	2,12
VIII. Ammoniumzitrat + Harnstoff	—	—	—	1,13	3,47	2,81
IX. Ammoniumsulfat + Asparagin	—	—	—	1,17	2,53	2,06
X. Ammoniumzitrat + Asparagin	—	—	—	1,28	3,15	2,50

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von drei Parallelbestimmungen.

End-pH erreicht wurde. Es wurde deshalb eine zweite Versuchsreihe mit dem *Aspergillus*-Stamm Groningen angestellt. Die Kulturflüssigkeit enthielt:

		%
Saccharose		10
Zitronensäure		1
K_2O	(K_2SO_4)	0,02
$MgSO_4$		0,03
Cu	($CuSO_4$)	0,00005
Zn	($ZnSO_4$)	0,0002
Fe	($FeSO_4$)	0,0005
Mn	($MnSO_4$)	0,0001
NaOH		0,2
P_2O_5	($NH_4H_2PO_4$)	in steigenden Mengen.

Die folgenden N-Verbindungen wurden pro Liter hinzugefügt:

I. 4 g Harnstoff
II. 4 g Harnstoff + 1 g Asparagin
III. 6 g Asparagin
IV. 6 g Ammoniumsulfat.

Die Ergebnisse sind in Tabelle VII vereint worden.

Tabelle VII.
Der Einfluss der Stickstoffquelle auf Myzelertrag und pH (*Aspergillus niger* Stamm Groningen).

N-Quelle	0,002% P_2O_5			0,006% P_2O_5			0,010% P_2O_5		
	Myz. Gew. in g [1]	Anfangs-pH	End-pH	Myz. Gew. in g [1]	Anfangs-pH	End-pH	Myz. Gew. in g [1]	Anfangs-pH	End-pH
I. Harnstoff	0,39	3,41	2,89	1,07	3,43	3,00	1,42	3,41	2,65
II. Harnstoff + Asparagin	0,42	3,62	3,05	0,97	3,62	3,02	1,23	3,64	2,77
III. Asparagin	0,39	3,59	2,86	1,02	3,61	2,72	1,50	3,61	2,43
IV. Ammoniumsulfat	0,40	3,57	2,88	1,17	3,62	2,24	1,58	3,62	1,84

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von drei Parallelbestimmungen.

Es zeigt sich, dass der Pilz sich mit Harnstoff sehr gut entwickelt hat; im Gegensatz zum Stamm POSCHENRIEDER nimmt hier das pH auch in den Kulturen mit Harnstoff ab. Diese Abnahme ist aber mit Ammoniumsulfat viel grösser, bei 0,01% P_2O_5 sogar 1,78, gegen 0,76 mit Harnstoff. Die Mischungen von Harnstoff mit Asparagin nehmen eine Zwischenstellung ein, so dass für unseren Zweck das teuere Asparagin fortgelassen werden kann.

Es ist anzunehmen, dass die pH-Abnahme in Gegenwart von Harnstoff zum grössten Teil verursacht wird durch die Bildung von organischen Säuren, während nebenbei das SO_4'' vom K_2SO_4 und $MgSO_4$ daran beteiligt sein wird.

Da Harnstoff durch die vom Pilz ausgeschiedene Urease in Ammoniak und Kohlensäure gespalten wird, ist es denkbar, dass bei Vergrösserung der Harnstoffmenge in der Nährflüssigkeit soviel Ammoniak ins Freie gesetzt wird, dass die gebildeten Säuren neutralisiert werden und das pH während des Wachstums besser konstant gehalten werden kann.

Deshalb wurde ein Versuch angestellt, wobei zu der genannten Nährlösung als N-Quelle 4, 5, 6 und 8 g Harnstoff pro Liter zugesetzt wurde (Tabelle VIII).

Tabelle VIII.

Der Einfluss von Harnstoff auf Myzelertrag und pH (*Aspergillus niger* Stamm Groningen).

N-Quelle	0,006% P_2O_5			0,010% P_2O_5		
	Myz. Gew. in g [1])	Anfangs-pH	End-pH	Myz. Gew. in g [1])	Anfangs-pH	End-pH
4 g Harnstoff	0,98	3,52	3,05	1,51	3,48	2,84
5 g Harnstoff	0,95	3,62	3,15	1,38	3,58	2,91
6 g Harnstoff	0,91	3,50	3,21	1,35	3,48	3,03
8 g Harnstoff	0,88	3,48	3,22	1,21	3,47	3,12

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von drei Parallelbestimmungen.

Das Myzelgewicht nimmt bei Anwesenheit von mehr Harnstoff ab, bei 0.01% P_2O_5 sogar um etwa 20%; die pH-Abnahme beträgt bei 8 g Harnstoff nur noch 0,26 und 0,35, was weniger ist, als infolge des geringeren Wachstums zu erwarten war. Weil die Nährlösung aber nicht mehr optimal war, wurde bei den folgenden Untersuchungen nur 0,4% Harnstoff hinzugefügt.

Dass hier im Gegensatz zu den mit Stamm POSCHENRIEDER erhaltenen Resultaten das pH der Flüssigkeit nicht in alkalischer Richtung verschoben wurde, ist vielleicht auf spezifische Unterschiede im Ureasegehalt der beiden Stämme zurückzuführen.

In wieweit die restierenden pH-Änderungen vom SO_4''-Teil des Kalium- und Magnesiumsulfats herrührten, wurde untersucht, indem diese Salze durch Zitrate ersetzt wurden.

Das Wachstum war bis einer Konzentration von 0,002% P_2O_5 normal, bei höherem Phosphatgehalt aber blieb die Entwicklung zurück, was wahrscheinlich auf Mangel an Schwefel zurückzuführen war. Es wurden deshalb einige verschiedene Schwefelverbindungen untersucht, nämlich Natriumthiosulfat, Thioharnstoff, Kalziumsulfat und Schwefel (Tabelle IX).

Tabelle IX.
Einfluss der Schwefelquelle auf die pH-Änderungen
(*Aspergillus niger* Stamm Groningen).

P_2O_5 in %	S-Verbindung	Myzelgewicht in g [1])	Anfangs-pH	End-pH
0,01	Ohne S	0,36	3,67	3,43
0,01	0,1% $Na_2S_2O_3$	0,33	4,63	3,99
0,01	0,2% $Na_2S_2O_3$	0,22	4,81	4,52
0,01	0,1% $CS(NH_2)_2$	1,07	3,86	3,18
0,01	0,1% $CaSO_4$	1,49	3,69	3,10
0,01	0,5% $CaSO_4$	1,72	3,64	2,76
0,01	0,05% S	0,79	3,67	3,60
	Normale Lösung mit K_2SO_4 und $MgSO_4$	1,42	3,41	2,65

[1]) Kulturzeit 4 Tage. Mittel von 3 Parallelbestimmungen.

Das $Na_2S_2O_3$ scheidet sofort aus, so dass es als S-Quelle unbrauchbar ist. Der S von Thioharnstoff hingegen ist ziemlich gut assimilierbar, obwohl die Entwicklung des Pilzes hinter derjenigen mit K_2SO_4 und $MgSO_4$ zurückbleibt. Auch Schwefel an sich wird vom Pilz assimiliert, doch nicht dermassen, dass das Myzel normal zur Entwicklung kommen könnte.

Das Verhalten des $CaSO_4$ ist bemerkenswert; mit 0,1% ist das Myzelgewicht demjenigen mit K_2SO_4 und $MgSO_4$ gleich, die pH-Änderung ist kleiner. Vergrössert man aber die Menge bis auf 0,5%, so steigt das Myzelgewicht bis zu 1,72, das pH sinkt aber von 3,64

bis auf 2,76. Es ist klar, dass der Pilz aus dem $CaSO_4$ nicht nur S aufgenommen hat, sondern auch Ca-Ionen assimiliert hat.

Das $CaSO_4$ bietet also als S-Quelle keine besondere Vorteile vor K_2SO_4 und $MgSO_4$, weshalb diese letzten Salze definitif in die Nährlösung aufgenommen wurden. Es war aber nötig, die Wirkung des Kalziums näher zu untersuchen.

5. DER EINFLUSS VON KALZIUM-VERBINDUNGEN AUF DIE ENTWICKLUNG VON *ASPERGILLUS NIGER.*

Schon im Jahre 1891 hatte WEHMER (20) darauf hingewiesen dass Ca-Ionen einen deutlich merkbaren Einfluss auf das Wachstum von *Aspergillus niger* ausüben. BUROMSKY (4), BENECKE (2) und MOLISCH(8) schreiben dem Kalzium, besonders in Gegenwart von Mg, einen günstigen Einfluss auf die Myzelbildung zu.

TRISCHLER (18) fand, dass durch Hinzufügung von Ca-Verbindungen das pH der Lösung sich während des Wachstums des Pilzes in alkalischer Richtung änderte. Auch von NIKLAS und Mitarbeitern (13) ist die Frage näher untersucht worden; sie halten es für notwendig, bei den Kaliumbestimmungen in kalkhaltigen Böden die Menge des darin anwesenden Kalziums zu berücksichtigen. In welcher Weise dies geschehen muss, wird aber nicht angegeben.

Wir haben über den Einfluss verschiedener Kalziumverbindungen auf die Entwicklung des Pilzes einige Versuche angestellt. Zur Nährlösung I auf Seite 93, welche als N-Quelle 0,6% Ammoniumsulfat enthielt, wurde bei verschiedenen P_2O_5-Konzentrationen steigende Mengen $CaCO_3$ hinzugefügt (Tabelle X).

Bei einer Menge von 0,75 g $CaCO_3$ pro Gefäss hat sich in keinem Falle Myzelium gebildet; mit 0,50 g $CaCO_3$ entwickelt sich nur bei der höchsten P_2O_5-Konzentration ein kräftiges Myzel, welches jedoch hinter demjenigen mit 0,25 g $CaCO_3$ zurückblieb. Dass es sich bloss um das pH handelte, war nicht wahrscheinlich, denn Nr. 3 mit einem Anfangs-pH von 6,13 hat kein Myzel gebildet, während Nr. 11 mit einem Anfangs-pH von 7,62 ein üppiges Wachstum zeigte.

Eine Menge von 0,50 g $CaCO_3$ in den 7,5 g Boden, welche zur Untersuchung gelangen, stimmt überein mit einem Gehalt von etwa 6,7% $CaCO_3$, was in der Praxis gar keine Ausnahme ist. Es ist also unumgänglich, das im Boden anwesende $CaCO_3$ zu neutralisieren, wie auch von NIKLAS angegeben worden ist; am Besten ist dazu die Zitronensäure geeignet.

Zur weiteren Untersuchung über die Rolle des Kalziums wurde

Tabelle X.

Der Einfluss steigender Mengen $CaCO_3$ auf Myzelgewicht und pH (*Aspergillus niger* Stamm Groningen).

P_2O_5 in %	$CaCO_3$ in g	Anfangs-pH	End-pH	Myzelgewicht in g [1])
0,002	0,0	3,53	2,95	0,33
	0,25	4,62	3,15	0,33
	0,50	6,13	6,52	0,00
	0,75	7,26	8,40	0,00
0 006	0,0	3,58	2,64	1,03
	0,25	4,68	3,22	0,92
	0,50	—	7,80	0,00
	0,75	7,76	8,20	0,00
0,010	0,00	3,53	1,65	1,66
	0,25	4,72	2,68	1,88
	0,50	7,62	3,19	1,65
	0,75	7,60	7,45	0,00

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von 3 Parallelbestimmungen.

Kalziumsulfat und -Zitrat herangezogen. Bei Hinzufügung vom Zitrat zur sauren Nährlösung TRISCHLER's steigt das pH von 2,5 bis etwa 3,4 wodurch NaOH-Zusatz unnötig wird. Aus der Tabelle XI ist ersichtlich, dass in allen Fällen Zusatz der Kalziumverbindungen eine deutliche Vermehrung der Myzelgewichte verursacht hat.

Tabelle XI.

Der Einfluss von $CaSO_4$ und Ca-Zitrat auf Myzelgewicht und pH (*Aspergillus niger* Stamm Groningen).

Ca-Verbindung	Anfangs-pH	End-pH	Myzelgewicht in g [1])
Kontrolle, ohne Zusatz	3,49	1,85	1,56
0,4 g $CaSO_4$	3,45	1,80	1,85
1,25 g $CaSO_4$	3,54	1,94	1,97
0,4 g Ca-Zitrat	3,31	1,98	1,89
1,25 g Ca-Zitrat	3,38	2,84	2,02

[1]) Kulturzeit 4 Tage bei 37°C. Nährlösung TRISCHLER mit 0,01 % P_2O_5.

Bemerkenswert ist, dass obwohl mit 1,25 g Zitrat das Myzelgewicht

am grössten ist, das pH sich am wenigsten geändert hat; es tritt hier die Pufferwirkung des Kalziumzitrats in den Vordergrund, weshalb es dem Sulfat vorzuziehen ist.

Die obige Nährlösung war diejenige TRISCHLER's mit Ammoniumsulfat als Stickstoffquelle; es war die Frage, wie gross die pH-Änderungen sein würden in der geänderten Nährlösung II (S. 93) mit Harnstoff als N-Quelle (Tabelle XII).

Tabelle XII.

Der Einfluss von Ca-Zitrat in Nährlosung II mit Harnstoff und 0,01% P_2O_5 auf Myzelgewicht und pH (*Aspergillus niger* Stamm Groningen).

Ca-Zitrat pro Gefäss in g	Anfangs-pH	End-pH	Myzelgewicht in g [1])
0,75	3,40	3,19	1,71
1,25	3,49	3,12	2,07
1,50	3,49	3,12	2,12
Ohne Zusatz	3,48	2,84	1,54

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von 3 Parallelbestimmungen.

Auch hier eine stetige Steigung des Myzelgewichts bei zunehmendem Ca-Gehalt; die pH-Änderung ist jetzt bis 0,37 zurückgebracht, gegen 0,64 ohne Kalziumzitrat-Zusatz und 1,78 in der Originalnährlösung TRISCHLER's. Da auch bei höherem P_2O_5-Gehalt Vermehrung des Zitrats über 1,25 g hinaus weder Myzelgewicht noch pH weiter beeinflusste, wurde beschlossen, bei den P_2O_5-Bestimmungen im Boden 1,25 g Kalziumzitrat pro Erlenmeyer hinzuzufügen.

Aus dem Obigen ist es klar geworden, dass der wechselnde Gehalt an $CaCO_3$ der zu untersuchenden Böden trotz der Neutralisierung mit Zitronensäure, das Wachstum des Pilzes auf wenigstens zwei Weisen beeinflussen kann, nämlich durch den Nährwert des Kalziums und durch die Pufferwirkung des gebildeten Kalziumzitrats. Es können dadurch Fehler von mehr als 40% entstehen, denen durch die oben beschriebenen Änderungen des Substrats vorgebeugt werden können.

6. DER EINFLUSS DES WUCHSSTOFFS RHIZOPIN UND VON HUMUSSÄURE AUF DAS WACHSTUM VON *ASPERGILLUS NIGER*.

NIELSEN (9) später auch zusammen mit HARTELIUS (10), hat gezeigt, dass ein von ihm aus dem Pilz *Rhizopus suinus* isolierter Wuchsstoff, das Rhizopin, das Wachstum des *Aspergillus niger* stimuliert.

Kiessling (6) konstatierte, dass Humuszusatz das Wachstum von *Aspergillus niger* förderte. Mit Rücksicht auf den stimulierenden Einfluss, den bisweilen das Beimischen von nur einigen Grammen Bodens auf die Entwicklung verschiedener Mikroorganismen ausübt, war es nicht ausgeschlossen, dass auch im Boden derartige Stoffe bisweilen vorkommen. Wenn dies der Fall wäre, so würde durch die An- oder Abwesenheit dieser Stoffe das Myzelgewicht mitbestimmt werden, was einer zuverlässigen Deutung der Phosphatbestimmungen im Wege stehen würde. Es war daher nicht ohne Bedeutung, über das Verhalten des Pilzes zu den fraglichen Stoffen orientiert zu sein.

Die Rhizopinlösung wurde nach der Vorschrift Nielsen's bereitet: *Rhizopus suinus* wurde auf einer Nährlösung gezüchtet, die pro Liter enthielt 10 g Ammoniumtartrat, 10 g Glukose, 0,5 g KH_2PO_4, 0,03 g $MgSO_4$ und die Mikroelemente Fe, Cu und Mn. Von dieser Lösung wurde 200 ccm in einen Liter-Erlenmeyerkolben gefüllt, sterilisiert und mit *Rhizopus suinus* geimpft. Nach 5 Tagen bei 37°C. wurde die Lösung filtriert, 15 Min. bei ½ Atm. sterilisiert und zur weiteren Untersuchung verwendet. Weder die mittels Alkohol-präzipitation gereinigte Lösung, noch der Aether-Extrakt übten eine förderende Wirkung auf das Wachstum von *Aspergillus niger* aus. Nur die ursprüngliche wässerige Lösung wirkte stimulierend.

Zur Untersuchung wurden steigende Mengen dieser Rhizopin-lösung zu 75 ccm Nährlösung I mit 0,008% P_2O_5 neutralisiert mit NaOH hinzugefügt (Tabelle XIII).

Tabelle XIII.
Der Einfluss von Rhizopin auf Myzelgewicht
(*Aspergillus niger* Stamm Groningen).

Menge der Rhizopin-Lösung	Myzelgewicht in g [1])
0	1,09
1 cc	1,23
5 cc	1,31
10 cc	1,25

[1]) Kulturzeit 3 Tage bei 37°C. Mittel von 3 Parallelbestimmungen.

Die Analyse der Rhizopin-Lösung ergab, dass sie 0,015 g P_2O_5 pro 100 ccm enthielt; durch die Hinzufügung van 1 ccm Rhizopin-Lösung zu 75 ccm Nährflüssigkeit wird der Phosphatgehalt also um 0,0002% gesteigert und auf 0,0082 gebracht. Die Myzelgewichts-

zunahme infolge dieser erhöhten Phosphatkonzentration beträgt maximal 0,05 g. Mit 1 ccm Rhizopin-Lösung war aber eine Myzelgewichtszunahme von 0,14 g erreicht worden, was also auf eine förderende Wirkung hinweist.

Bei einem zweiten Versuch wurde die Wirkung des Rhizopins mit derjenigen einer kleinen Menge Humussäure verglichen. Gebraucht wurde Humussäure Merck, aus Zucker bereitet und eine selbst aus Torf hergestellte Humussäure, welche beide unter Zusatz von ein wenig NaOH gelöst waren. Die Preparate waren P_2O_5-frei (Tabelle XIV).

Tabelle XIV.

Der Einfluss von Rhizopin und Humussäure auf Myzelgewicht (*Aspergillus niger* Stamm Groningen).

Zugefügte Substanz	Myzelgewicht in g [1])
Nährlösung II mit 0,01% P_2O_5, ohne Zusatz	1,17
mit 1 ccm Rhizopin-Lösung	1,40
mit 12,5 mg Humussäure (Merck)	1,25
mit 12,5 mg Humussäure (Torf)	1,38

[1]) Kulturzeit 3 Tage bei 37°C. Mittel von 3 Parallelbestimmungen.

Dieser Versuch bestätigt den Einfluss des Rhizopins, während auch die Humussäure stimulierend wirkt. Zur weiteren Orientierung wurde ein dritter Versuch angestellt, wobei auch die pH-Änderungen gemessen wurden (Tabelle XV).

Tabelle XV.

Der Einfluss von Rhizopin und Humussäure auf Myzelgewicht und pH (*Aspergillus niger* Stamm Groningen).

Zugefügte Substanz	Myzelgewicht in g [1])	Anfangs-pH	End-pH
Nährlösung II [2]) mit 0,01% P_2O_5 ohne Zusatz	1,35	4,17	3,37
mit 1 ccm Rhizopin-Lösung	1,60	4,18	3,38
mit 12,5 mg Humussäure (Merck)	1,83	4,18	3,37
mit 12,5 mg Humussäure (Torf)	1,91	4,18	3,40
mit 12,5 mg Humussäure + 1 ccm Rhizopin-Lösung	1,94	4,18	3,40

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von 4 Parallelbestimmungen.

[2]) Irrtümlich noch mit 0,2% NaOH neutralisiert, deswegen Anfangs-pH 4,18 statt 3,5.

Die Myzelgewichtssteigerung bei Zusatz von Humussäure übertrifft diejenige des Rhizopins, während bei Mischung der beiden Substanzen das Myzelgewicht nicht mehr zunimmt [1]).

Die Zunahme durch die Humussäure ist recht beträchtlich und beträgt etwa 42%. Es besteht also die Möglichkeit, dass ein Boden, ausser dem zu bestimmenden Phosphat, Stoffe enthält, die das Wachstum des Pilzes beschleunigen; dieser störende Einfluss lässt sich leicht beseitigen, indem zu der Nährlösung eine kleine Menge Humat zugesetzt wird. Zur weiteren Bestätigung des Obigen wurde an Böden von verschiedener Herkunft und Zusammensetzung eine Bestimmung mit und ohne Zusatz von Humussäure unternommen (Tabelle XVI).

Tabelle XVI.

Der Einfluss von Humussäure auf Myzelgewicht bei Anwesenheit von Böden verschiedener Herkunft (*Aspergillus niger* Stamm Groningen).

Boden	Myzelgewicht in g [1])		Mitt. Fehler d. Bestimm.	Differenz d. Humuszusatz
	Ohne Humus	Mit Humus		
Leichter Lehmboden	0,42	0,53	±4,8%	+26,2%
Heide-sand	0,44	0,44	4,5	0
Dasselbe, mit Phosphat gedüngt	1,14	1,11	2,7	— 2,7
Lehmboden ungedüngt	0,54	0,76	3,7	+40,7
Lehmboden	0,66	0,73	1,5	+ 9,1
Dünensand	0,51	0,53	5,9	+ 4,0
Lehmboden	1,34	1,33	5,3	— 0,8
Humoser Lehm	0,42	0,58	4,8	+38,1

[1]) In Erlenmeyerkolben von 300 ccm wurde an 75 ccm Nährlösung II ohne Phosphat, 7,5 g Boden hinzugefügt. Kulturzeit 4 Tage bei 37°C. Mittel von drei Parallelbestimmungen.

Es geht aus diesem Versuch deutlich hervor, dass es in der Tat Böden gibt, bei denen ein Humatzusatz förderend wirkt auf das erzeugte Myzelgewicht. Es gibt Zunahmen von 9.1 bis 40.7%, die weit über die Fehlergrenze hinaus gehen. Dass es daneben Böden gibt, die nicht auf Humatsuzatz reagieren, war zu erwarten; derartige Böden enthalten offenbar die stimulierenden Stoffe schon selbst. Die Anzahl der

[1]) Die Frage nach der eigentlichen Ursache der Stimulierung durch Rhizopin überschreitet den Rahmen dieser Untersuchung, um so mehr als dieser Stoff durch Humat ersetzt werden konnte.

untersuchten Böden ist zu klein, um näheres über den Zusammenhang zwischen Bodenart und Stimulation angeben zu können.

Es ist jedenfalls erwiesen, dass es zu empfehlen ist, bei der Bodenuntersuchung für die Praxis die Nährflüssigkeit durch Humatzusatz zu komplettieren und damit eine unkontrollierbare Fehlerquelle zu beseitigen.

7. WACHSTUM UND pH-ÄNDERUNGEN BEI STEIGENDEM PHOSPHATGEHALT DES KULTURMEDIUMS.

Mit Rücksicht auf eine richtige Interpretation des Myzelgewichts bezüglich des Phosphatgehalts der Nährflüssigkeit, war es erwünscht zu wissen, wie sich der Phosphatgehalt des Pilzmyzels bei zunehmender Phosphatkonzentration änderte.

Zu diesem Zweck wurden zur Nährlösung II steigende Mengen Ammoniumphosphat hinzugefügt; das Myzel wurde nach 4 tägiger Kultivierung bei 37°C. geerntet, tüchtig abgespült, getrocknet und gewogen. Dann wurde es nach A. NEUMANN auf nassem Wege verascht mittels eines Gemisches von gleichen Teile konzentrierter Schwefelsäure und Salpetersäure (1). In der Lösung der Asche wurde die Phosphorsäure nach LORENZ als Ammoniumphosphormolybdat bestimmt (Tabelle XVII).

Tabelle XVII.

Phosphatgehalt des Myzels bei steigendem Phosphatgehalt des Kulturmediums (*Aspergillus niger* Stamm Groningen).

P_2O_5-Gehalt des Kulturmediums in %	Myzelgewicht in g.[1])	P_2O_5-Gehalt des Myzels in %
0,00	0,00	0,00
0,002	0,29	0,31
0,004	0,61	0,30
0,006	0,95	0,28
0,008	1,27	0,28
0,010	1,55	0,26
0,012	1,90	0,24
0,014	2,14	0,23
0,016	2,29	0,23
0,020	2,45	0,27
0,024	2,74	0,27
0,026	2,78	0,30

[1]) Kulturzeit 4 Tage bei 37°C. Mittel von drei Parallelbestimmungen.

Bemerkenswert ist, dass der Phosphatgehalt des Myzels am höchsten war bei der kleinsten P_2O_5-Konzentration in der Nährflüssigkeit.

Es ist nicht ausgeschlossen, dass dieses eigentümliche Verhalten verursacht wird durch die Tatsache, dass das Verhältniss von Sporenmaterial zu Myzel bei den niedrigsten P_2O_5-Konzentrationen des Kulturmediums am grössten ist, vorausgesetzt, dass der P_2O_5-Gehalt der Sporen grösser ist als derjenige des Myzels.

Bei zunehmender Phosphat-Konzentration nimmt der P_2O_5-Gehalt des Myzels ständig ab, um, sobald die Myzelgewichtskurve nach rechts umbiegt, ein Minimum zu erreichen und nachher wieder zu steigen (Fig. 1).

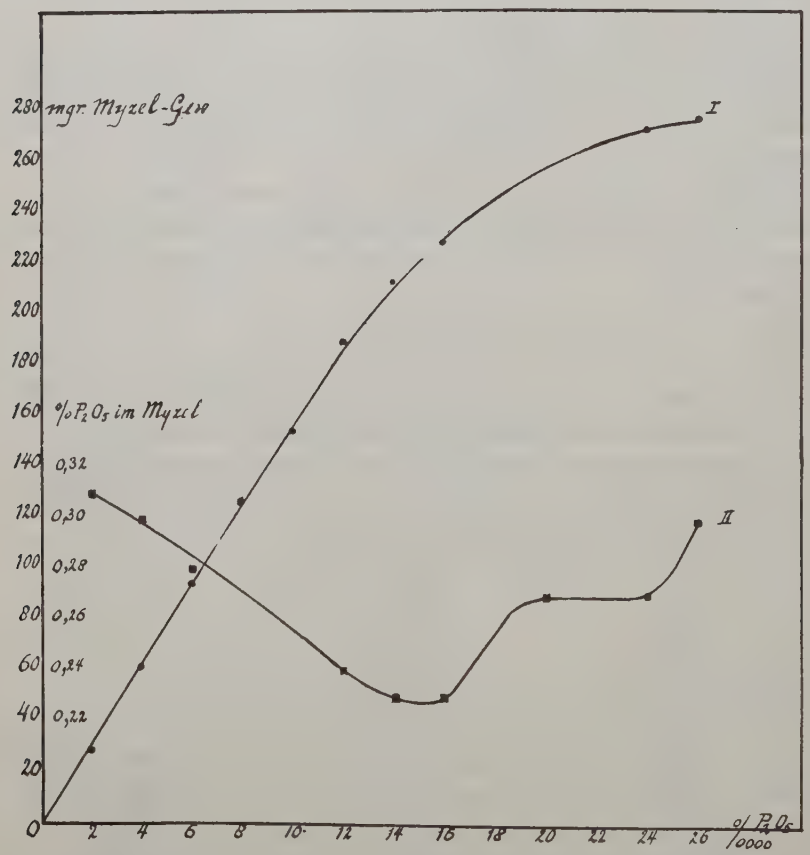

Fig. 1. Myzelgewichte (I) und die korrespondierenden Phosphatprozentsätze des Myzels (II) bei steigendem Phosphatgehalt der Nährlösung.

Obwohl dieses Verhalten ziemlich kompliziert ist, zeigt die Kurve, welche das Verhältnis zwischen P_2O_5-Gehalt des Kulturmediums und Myzelgewicht angibt, einen ziemlich regelmässigen Verlauf. Am einfachsten wäre es also, diese Kurve auf experimentellem Wege zu bestimmen und nachher bei den Phosphatbestimmungen in Böden den zum bestimmten Myzelgewicht gehörenden Phosphatgehalt aus der Kurve abzulesen.

8. DIE ERTRAGSKURVE.

Zu diesem Zweck wurde die Phosphat-Myzelgewichtskurve so genau wie möglich bestimmt; es wurde dabei der Stamm POSCHENRIEDER auf die übliche Nährlösung TRISCHLER's mit dem Stamme Groningen auf die abgeänderte Nährlösung II verglichen. Zugleich wurden die korrespondierenden pH-Änderungen bestimmt.

Die Nährlösungen hatten die folgende Zusammensetzung:

	Nährlösung I (TRISCHLER) (etwas geändert)	Nährlösung II (Groningen)
	%	%
Saccharose	10	10
Zitronensäure	1	1
Pepton	0,1	
$(NH_4)_2SO_4$	0,6	
Harnstoff		0,4
K_2O (K_2SO_4)	0,02	0,02
$MgSO_4$	0,03	0,03
Cu ($CuSO_4$)	0,00005	0,00005
Zn ($ZnSO_4$)	0,0002	0,0002
Fe ($FeSO_4$)	0,0005	0,0005
Mn ($MnSO_4$)	0,0001	0,0001
Ca-Zitrat		1,25 g [1]
Humussäure (als Na-Humat)		12,5 mg [1]
pH	± 2,5	± 3,5

[1]) pro 75 ccm.

Zu beiden Lösungen wurden steigende Mengen $NH_4H_2PO_4$ hinzugefügt. Die Erlenmeyerkolben von 300 ccm enthielten 75 ccm Nährlösung, es wurden immer 4 Parallelbestimmungen angesetzt und pünktlich 96 Stunden bei 37°C. kultiviert. Das Myzel entwickelte sich regelmässig, das Wachstum war besonders bei den höheren P_2O_5-

Konzentrationen üppig, alle Kulturen waren am Ende des Versuchs mit Sporen bedeckt (Tabelle XVIII und Fig. 2).

Tabelle XVIII.

Die Bestimmung der Ertragskurve bei steigendem Phosphatgehalt der Nährlösung.

P_2O_5 in %	Stamm Groningen			Stamm Poschenrieder		
	Myz. Gewicht in g	Anfangs-pH	End-pH	Myz. Gewicht in g	Anfangs-pH	End-pH
0	0.005 ± 0.00	3.55	3.33	0.05 ± 0.00	2.53	2.53
0.001	0.21 ± 0.024	3.55	3.29	0.16 ± 0.036	2.53	2.53
0.002	0.39 ± 0.00	3.53	3.29	0.33 ± 0.021	2.53	2.39
0.003	0.58 ± 0.020	3.53	3.36	0.50 ± 0.013	2.53	2.27
0.004	0.82 ± 0.046	3.53	3.45	0.65 ± 0.012	2.53	2.15
0.005	0.96 ± 0.074	3.53	3.45	0.75 ± 0.023	2.53	2.04
0.006	1.15 ± 0.026	3.53	3.45	0.98 ± 0.032	2.53	1.84
0.007	1.29 ± 0.029	3.53	3.47	1.18 ± 0.071	2.53	1.68
0.008	1.52 ± 0.048	3.53	3.45	1.33 ± 0.038	2.53	1.58
0.009	1.61 ± 0.088	3.55	3.45	—	—	—
0.010	1.81 ± 0.048	3.53	3.45	1.72 ± 0.018	2.53	1.58
0.012	2.17 ± 0.016	3.53	3.29	2.04 ± 0.010	2.53	1.54
0.014	2.31 ± 0.069	3.53	3.19	2.12 ± 0.020	2.53	1.57
0.017	2.66 ± 0.062	3.53	3.19	2.39 ± 0.022	2.53	1.52
0.020	2.75 ± 0.069	3.53	3.24	2.51 ± 0.037	2.53	1.52
0.025	—	—	—	2.49 ± 0.048	2.53	1.49
0.030	—	—	—	2.58 ± 0.033	2.53	1.49
0.040	2.45 ± 0.073	3.53	4.00	—	—	—

In beiden Fällen nehmen die Myzelgewichte regelmässig zu, diejenige des Stammes Poschenrieder bleiben etwas hinter den anderen zurück. Es sei darauf hingewiesen, dass der mittlere Fehler der Bestimmungen klein ist und im Allgemeinen nicht mehr als einige Prozente beträgt; die Reproduzierbarkeit der Bestimmungen übertrifft bei weitem diejenige der Neubauer-Bestimmungen und ist, wie aus anderen hier nicht genannten Untersuchungen hervorgeht, derjenigen der chemischen Bestimmungen in Böden fast gleichwertig.

Bei Betrachtung der pH-Zahlen kommt der Unterschied zwischen dem Verhalten der beiden Nährlösungen erst deutlich zu Tage: in der üblichen Nährlösung mit dem Stamm Poschenrieder sinkt das pH schon bei einer Konzentration von 0,008% P_2O_5 um fast 1,0 auf pH 1,58 herab; mit dem Stamm Groningen auf Nährlösung II beträgt

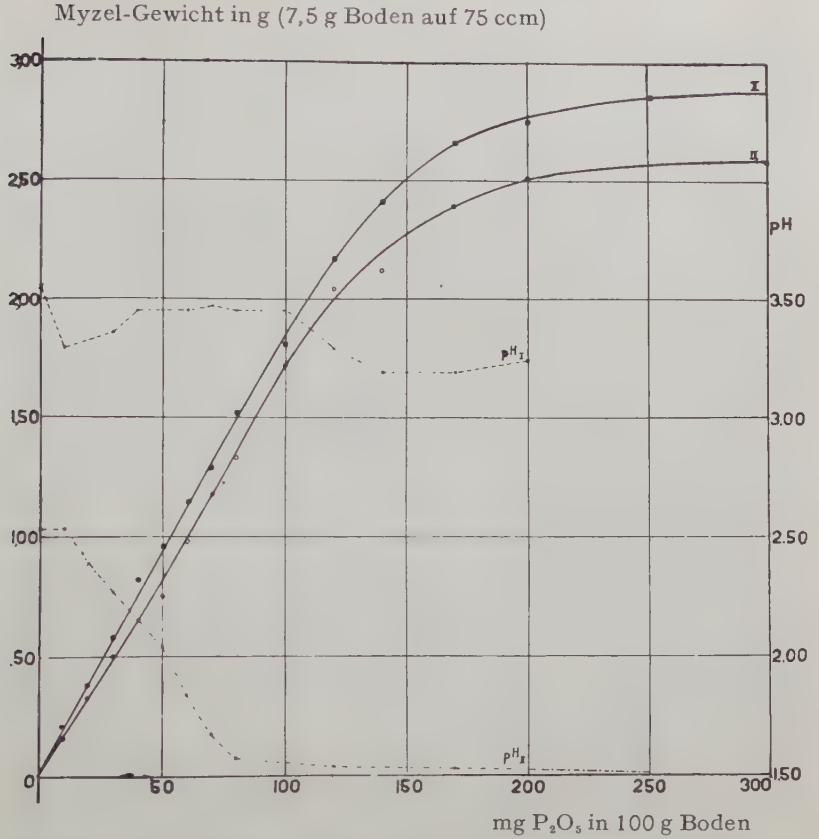

Fig. 2. Ertragskurven bei steigenden Phosphatgehalt:
I. Von *Aspergillus niger* Stamm Groningen auf Nährlösung II.
II. Von *Aspergillus niger* Stamm POSCHENRIEDER auf Nährlösung I.
Die gestreichelten Kurven geben die zugehörigen pH-Änderungen an.

die pH-Abnahme nur noch 0,08. Bis zu einer P_2O_5-Konzentration von 0,01% sind Anfangs- und End-pH praktisch gleich geblieben; von 0,012 bis 0,02% P_2O_5 tritt eine kleine Abnahme ein, welche aber nirgendwo mehr als 0,35 pH beträgt. Dass bei 0,04% P_2O_5 das pH bis auf 4,0 gestiegen ist, wird durch Ammoniakbildung verursacht, entweder durch die enzymatische Spaltung von Harnstoff durch die Urease oder durch Proteolyse des Pilzmyzels. Auf letzteres weist auch die Abnahme des Myzelgewichts gegenüber dem Versuch mit 0,02% P_2O_5 hin. Eine derartig hohe Konzentration kommt aber bei den Bodenuntersuchungen fast nie vor.

Die pH-Änderungen, die in Nährlösung I auftreten können, sind

bei Hinzufügung von Böden beträchtlich grösser, weil durch die im Boden anwesenden Basen das Anfangs-pH über pH 3,5 hinaus steigen kann.

Dass es auch bei der Phosphatbestimmung in Böden gelingt, mit Hilfe der abgeänderten Nährlösung II und mit dem *Aspergillus niger* (Stamm Groningen) die pH's innerhalb enger Grenzen konstant zu halten, geht aus der Tabelle XIX deutlich hervor. Wichtig ist auch, dass bei den verschiedenen Böden, ungeachtet ihres Gehaltes an $CaCO_3$ oder anderer Basen, das Anfangs-pH praktisch dasselbe ist.

Tabelle XIX.

Nr.	Boden	% $CaCO_3$	Myz. Gew. in g	Anf.-pH	End-pH	Diff.
1	Heidesand, ungedüngt	0	0,09	3,41	3,41	0,00
2	Dasselbe, mit 100 Kg P_2O_5 pro H.A. gedüngt	0	0,21	3,40	3,40	0,00
3	Lehmboden	0,14	0,38	3,62	3,32	0,30
4	Dasselbe, mit Superphosphat gedüngt	0,14	0,50	3,62	3,32	0,30
5	Lehm-Grasland	—	0,46	3,57	3,29	0,28
6	Sandiger Lehm	2,8	0,61	3,67	3,67	0,00
7	Lehm-Grasland (= Nr. 5) aber mit 150 Kg P_2O_5 pro H.A. gedüngt	—	0,78	3,59	3,36	0,23
8	Alter Mohrboden, ungedüngt	0	0,83	3,43	3,41	0,02
9	Kalkhaltiger Lehmboden	4,4	1,22	3,81	3,81	0,00
10	Alter Mohrboden (= Nr. 8) regelmässig Stallmisst	0	1,53	3,45	3,29	0,16
11	Heidesand (= Nr. 1) mit schwerer Phosphatdüngung	0	1,53	3,41	3,33	0,08
12	Sand, Grasland	—	1,61	3,55	3,36	0,19
13	Kalkreicher See-Ton	10,24	1,29	3,74	3,74	0,00
14	Alter Mohrboden (= Nr. 8) regelmässig Volldüngung	0	1,83	3,45	3,29	0,16
15	Sand-Grasland (= Nr. 12) mit 150 Kg P_2O_5 pro H.A. gedüngt	—	2,57	3,59	3,17	0,42

Die pH-Änderungen sind im allgemeinen recht unbedeutend und betragen im Mittel nur 0,14 pH. Es sind gerade die kalkreichsten Böden, welche die kleinsten pH-Änderungen aufweisen, was darauf hinweist, dass es vielleicht möglich wäre das Kulturmedium noch besser zu puffern.

Jedenfalls ist bewiesen, dass mit Hilfe unseres *Aspergillus niger*-Stammes Groningen und der von uns geänderten Nährlösung die im

Anfang gestellte Aufgabe, eine optimale, gut gepufferte Nährlösung, deren Erträge vom $CaCO_3$-Gehalt des Bodens unabhängig sind, praktisch gelöst ist und deswegen der Anwendung der Methode zur Phosphatbestimmung in Böden keine prinzipiellen Einwände mehr im Wege stehen.

Zusammenfassung.

An der Bestimmung des Phosphatbedürfnisses von Ackerböden mit Hilfe von *Aspergillus niger* haften gewisse Schwierigkeiten, welche der praktischen Anwendung dieser raschen und billigen Methode im Wege stehen.

Infolge der wachsenden Säurebildung durch den Pilz bei steigenden Phosphatmengen in der Nährlösung und ihren geringen Pufferkapazität ist bei der Methode von Niklas und seinen Mitarbeitern das pH, bei dem die Böden extrahiert werden, nicht konstant, sondern weitgehend vom Phosphatgehalt dieser Böden abhängig.

Daneben beeinflusst der wechselnde Kalkgehalt der Böden das Wachstum des Pilzes auf zwei Wegen, erstens durch die Änderung der Pufferung des Kulturmediums und zweitens, weil Kalzium für den Pilz ein Nährstoff ist und in der Nährlösung in ungenügender Menge anwesend ist. Auch die An- oder Abwesenheit bestimmter stimulierender humöser Stoffe beeinflusst die Menge des gebildeten Myzels.

Es zeigte sich bei unseren Untersuchungen, dass man die pH-Änderungen beträchtlich herabsetzen kann, wenn statt 0,6% Ammoniumsulfat, 0,4% Harnstoff als Stickstoffquelle gebraucht wird.

Eine von uns isolierter *Aspergillus niger*-Stamm zeigte den Vorteil, dass er sich mit Harnstoff und ohne Pepton gut entwickelte, im Gegensatz zu dem bisher gebrauchten *Aspergillus niger*-Stamm, der zur üppigen Entwicklung Ammoniumsulfat und Pepton braucht.

Durch Hinzufügung von Kalziumzitrat zur Nährlösung wurde zugleich eine bessere Pufferung und eine weitgehende Unabhängigkeit vom Ca-Gehalt des Bodens erreicht.

Durch Zusatz von sehr wenig Humussäure (als Na-Humat) wurde eine Quelle von Unregelmässigkeiten beseitigt; in einigen Fällen wurde dadurch 9 bis 40% mehr Myzel gebildet, in anderen Fällen hatte Humatzusatz gar keinen Einfluss.

Durch diesen Massnahmen wurden die pH-Änderungen von maximal 1,8 pH bis auf 0,37 zurückgedrängt. Bei der Untersuchung einer Anzahl Böden von verschiedener Herkunft betrugen die pH-Änderungen im Mittel nur noch 0,14 pH.

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(From the Microbiological Laboratory, Agricultural University, Wageningen.)

ON THE USE OF MICRO-ORGANISMS IN MEASURING A DEFICIENCY OF COPPER, MAGNESIUM AND MOLYBDENUM IN SOILS[1])

by

E. G. MULDER.

(Received January 26, 1940.)

1. INTRODUCTION.

The fact that micro-organisms may be used in determining the amount of plant food in soils, results from their faculty to assimilate, generally speaking, the same foodstuffs as the higher plants. In adding a certain quantity of soil to a liquid medium, free of a certain nutrient, the rate of growth of a test organism, inoculated in this medium, is a measure of the amount of this particular nutrient present in the soil in an available form. In comparison with chemical analysis microbiological tests show the advantage that estimation of a certain element is possible without separating it from other compounds. This saves a great amount of time especially in the case of a determination of the so-called minor elements, such as copper, zinc, molybdenum etc. Another advantage is that the same organism and similar methods may be used in determining almost all of the elements essential for plant growth.

In the *Aspergillus niger* tests for potassium, phosphorus and magnesium, according to NIKLAS and collaborators (10), the fungus is cultivated in 75 ml Erlenmeyer flasks containing 30 ml of a nutrient liquid medium. The development of the fungus, estimated by weighing the mycelia, is a measure of the amount of plant-available potassium, phosphorus and magnesium, respectively.

In order to avoid the necessity of weighing the mycelia, 1000 ml Erlenmeyer flasks containing 40 ml of a nutrient solution were used

[1]) Presented at the meeting of the Netherlands Society of Microbiology, Wageningen, May 13th, 1939.

in the determination of available copper and magnesium in soils. In these large flasks the *Aspergillus* mycelia have about 10 times the surface of those in the 75 ml vessels. This results in much more significant differences in the appearance of the mycelia and spores by increasing amounts of copper and magnesium, than in the case of the small flasks. Since these differences are reproducible, it is possible to estimate the concentration of these elements in the soil without weighing the mycelia only by comparing the cultures to which soil is given with those of a standard series, to which increasing amounts of copper and magnesium are added.

2. COPPER TESTS.

In the *Aspergillus* test for determining the copper which was recently worked out and described (6, 7, 8. Cf. also the earlier investigations of WOLFF and EMMERIE (12)), a nutrient solution of the following composition is used:

Water distilled from Jena glassware 1000 ml

Glucose	50 g	$FeCl_3 . 6H_2O$	0.050 g
KNO_3	5 g	$ZnSO_4 . 7H_2O$	0.020 g
K_2HPO_4	2.5 g	$MnSO_4 . 4H_2O$	0.003 g
$MgSO_4 . 7H_2O$	1 g	$Na_2MoO_4 . 2H_2O$	0.0015 g

In order to remove the small traces of copper which are found even in the purest commercial chemicals, 0.3 ml of a solution of NH_4SH and 5 g of charcoal (Norit) are added to the solution. After some 5 minutes' shaking the charcoal is separated by filtration. In order to avoid losses of the minor elements by adsorption on the charcoal, the iron, zinc and manganese salts and the molybdate are added after the filtration. Of this nutrient medium 40 ml portions are used in 1000 ml Erlenmeyer flasks of Jena glass. After sterilization of the solution for 5 minutes at 105°C., 1 gram of air-dry soil is added. The flasks are inoculated with a few drops of a suspension of *Aspergillus* spores in sterile, copper-free water and incubated at 31°C. After 4—5 days the amount of available copper can be estimated by comparing the colour of the mycelia with those of a standard series containing copper as sulphate in amounts of 0, 0.1, 0.2, 0.4, 0.6, 1, 1.3, 1.6, 2 and 2.5 γ Cu respectively [1]). Without copper the mycelia of *Aspergillus niger* are but insuffi-

[1]) It is advisable, in order to avoid the adsorption of the copper on the glass surface, to add the copper sulphate after the autoclaving of the solution.

ciently developed and quite sterile; increasing amounts of copper result in a more abundant spore formation, whereas the colour of the spores is changing from yellow to yellow-brown, gray-brown, black-brown and black (see the coloured plates in 6). Since the differences in colour of the mycelia of the standard series are clearly perceptible, the estimation of the available soil-copper may be made with a rather high degree of accuracy.

In these investigations a certain strain called "M" of *Aspergillus niger* was used throughout. Of 4 other strains two gave about the same results, the others, however, showed a different colour scale on account of increasing amounts of copper. So every strain has to be considered individually.

3. RESULTS OBTAINED WITH THE *ASPERGILLUS* TEST IN THE DETERMINATION OF COPPER.

Some 60 sandy and peaty soils from different parts of Holland were tested on their copper content. These estimations were carried out in connection with the investigation of the so-called "reclamation disease", occurring in many countries on peaty and sandy soils (6). This disease may be cured by the addition of copper sulphate in amounts of 50—100 pounds per acre. Cereals, especially oats, wheat and barley are very sensitive to the disease. The most typical symptoms are the rolled and shrunken youngest leaves and the white tips of the second highest leaves (fig. 1).

It appeared that soils on which the plants suffered from the disease had a very low content of available copper; soils producing healthy plants always showed much higher figures. Part of the results are recorded under Table I.

The data of Table I conform with the results of water culture experiments with cereals, according to which the symptoms of copper deficiency and those of the "reclamation disease" are identical (figures 1 and 2).

From these results and from copper analysis in healthy and diseased plant material it is concluded that the "reclamation disease" is caused by a deficiency of available copper.

Table I.

Available copper present in soils.

Soil	Plant growth	Available copper in γ per 1 g of soil
Sandy soil	wheat, healthy	> 2.5
„ „	white oats, healthy	> 2.5
„ „	„ „ , severely diseased	0.1
„ „	„ „ , slightly diseased	1.1
„ „	„ „ , healthy	1.5
„ „	„ „ , „	> 2.5
„ „	„ „ , diseased	0.3
peat soil	*a*) wheat, severely diseased	0.2
„ „	*b*) healthy spot in diseased field	2—2.5
„ „	wheat, severely diseased	0.2
sandy soil	„ , healthy	> 2.5
„ „	canary grass, healthy	> 2.5
peat soil	wheat, healthy	> 2.5
„ „	„ , „	> 2.5
sandy soil	„ , „	> 2.5
„ „	white oats, healthy	1.8
„ „	„ „ , diseased	0.4
peat soil	„ „ , severely diseased	0.1
sandy soil	„ „ , „ „	0.2
„ „	*a*) white oats, severely diseased	0.25
same field	*b*) „ „ , less diseased spot	0.8
„ „	*c*) „ „ , healthy spot	1.7
„ „	*d*) „ „ , cured by the addition of $CuSO_4$	> 2.5

4. MAGNESIUM TESTS.

For the determination of available magnesium in soils, a medium of the following composition is used:

Distilled water	1000 ml	$FeCl_3 . 6H_2O$	0.02 g
Glucose	50 g	$ZnSO_4 . 7H_2O$	0.01 g
KNO_3	5.0 g	$MnSO_4 . 4H_2O$	0.003 g
K_2HPO_4	2.5 g	$Na_2MoO_4 . 2H_2O$	0.0015 g
$Na_2SO_4 . 10H_2O$	1.0 g	$CuSO_4 . 5H_2O$	0.001 g

Three-gram portions of the air-dry soils are added to 1000 ml Erlenmeyer flasks containing 40 ml of the sterilized medium, and the 5-day-old cultures are compared with those of a standard series to

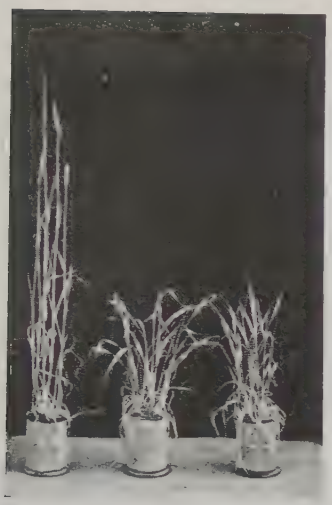


Fig. 1. "Reclamation disease" in barley. Left with the addition of 25 mg of copper sulphate per jar (100 pounds per acre).

Fig. 2. Copper deficiency in barley. Right: without copper, left: with the addition of 0.2 mg of copper sulphate per 1000 ml of nutrient solution.

which magnesium sulphate is added in amounts of 0, 25, 50, 75, 100, 150, 200, 300, 400 and 500 γ of magnesium, respectively. Up to 100 γ no spores are formed in these standards, but there are significant differences in the thickness of the mycelia. With 150 γ a slight spore formation is shown, which gradually grows with increasing amounts of magnesium. Between the 400 γ and 500 γ cultures only very slight differences are observed. The appearance of these standard cultures is not changed by adding calcium salts to the medium or by changing the K:Na ratio.

For this determination other organisms than *Aspergillus niger* may be used. Good results were obtained with *Azotobacter chroococcum*. The bacterium was grown on a medium of the following composition:

Distilled water 1000 ml			
Agar	20 g	$ZnSO_4 . 7H_2O$	0.001 g
Glucose	20 g	$MnSO_4 . 4H_2O$	0.0005 g
K_2HPO_4	1 g	$Na_2MoO_4 . 2H_2O$	0.0005 g
$Na_2SO_4 . 10H_2O$	0.2 g	$FeCl_3 . 6H_2O$	0.010 g
$CaCO_3$	1 g	$CuSO_4 . 5H_2O$	0.0005 g

For the purification of the agar from magnesia the following method

was used (3). The dry agar was cut into small pieces and covered with a solution of 10 per cent NaCl, which was refreshed after 10 hours. This treatment was repeated 10 times, after which the agar was washed with distilled water until no more Cl-ions could be detected.

Ten ml of this medium were mixed with 2 g of soil, boiled for some minutes and poured into 9 cm Petri dishes. After solidification the agar-soil-plates were covered with 5 ml of the same medium in order to get a smooth surface, and inoculated with a young culture of *Azotobacter chroococcum*. It appeared that without the addition of magnesium *Azotobacter* did not grow; with 10 γ of magnesium small colonies were formed, which much increased in size when 100 γ was given. The best growth, however, was obtained with 400 γ of magnesium per plate.

Another organism which may be used for the determination of small amounts of magnesium is *Bact. prodigiosum* which needs this element for its growth and for the development of its red pigment. In order to cultivate this bacterium an agar medium of the following composition was used:

Distilled water	1000 ml	$Na_2CO_3 . 10H_2O$	1 g
Agar	20 g	$ZnSO_4 . 7H_2O$	0.0005 g
Glucose	5 g	$FeCl_3 . 6H_2O$	0.001 g
K_2HPO_4	1 g	$MnSO_4 . 4H_2O$	0.0005 g
NH_4NO_3	1 g	$Na_2MoO_4 . 2H_2O$	0.0005 g
K_2SO_4	1 g	$CuSO_4 . 5H_2O$	0.0005 g

Plates, 9 cm in diameter, were prepared and inoculated with the bacterium. After 4 days at 20°C. very tiny, white colonies developed, due to the traces of magnesium present in the nutrient salts. With increasing amounts of magnesium a better growth appeared and the colour of the colonies gradually grew more reddish. Whether *Bact. prodigiosum* may be used for the estimation of the available magnesium in soils still remains to be investigated.

5. RESULTS DERIVED FROM THE MAGNESIUM TESTS.

Magnesium was determined in a number of sandy soils on which plants were suffering from the so-called "Hooghalen disease" or acidity disease, occurring especially on acid soils. The plants being very chlorotic are of poor growth. In cereals, dark green spots are shown on the yellow-green leaves: evidently the production of chlorophyl does not function properly.

Although a distinct relation is observed between the occurrence of the disease and the low pH of the soil, the latter is not the direct cause of the former. This is proved by cultivating plants in water or sand cultures provided with all of the necessary elements. A good development is obtained at pH-values which are lower than those of the soils on which the disease is found [1]).

In the publications of GEHRING (4) and VAN ITALLIE (5) good results of the application of magnesium salts to acid soils are shown. According to GEHRING the magnesium in these soils is very strongly adsorbed by the soil colloids and a neutralization of the soil is said to enhance the availability of the magnesium. VAN ITALLIE reports that plants growing on acid soils need more magnesium than those on neutral ones, whereas the adsorption of this nutrient by the roots does meet with more difficulties in the former case.

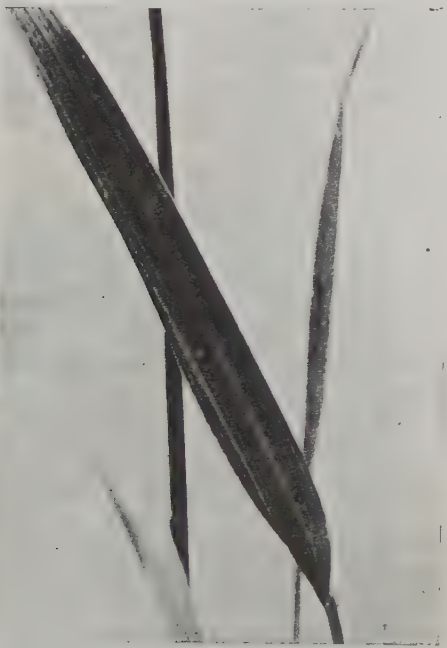


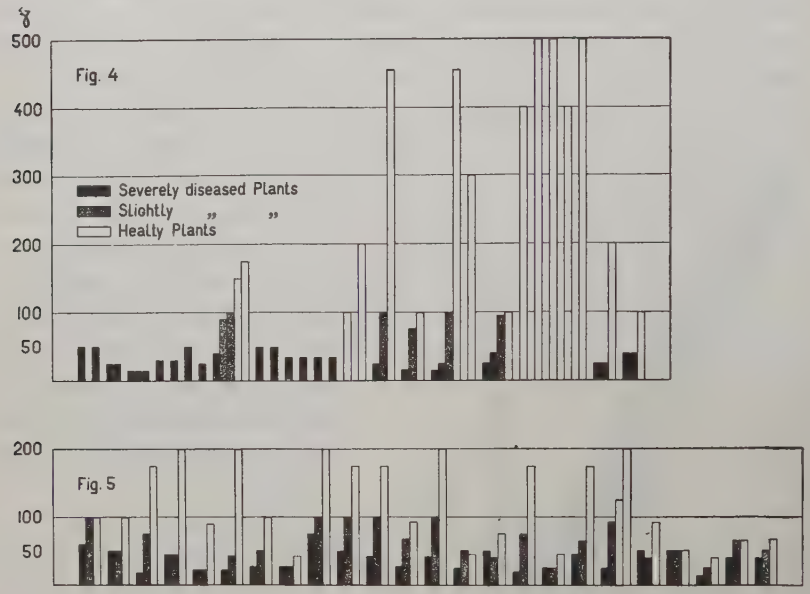
Fig. 3. Magnesium deficiency symptoms (white oats in a nutrient solution, a few days after the addition of a small amount of $MgSO_4$).

In studying the symptoms of magnesium deficiency in water cultures of cereals it appeared that these symptoms were very similar to those of the "Hooghalen disease". In both cases the plants grew poorly, with symptoms of chlorosis and showed dead lower leaves. The spotted leaves, which represent a milder form of the disease, could be obtained in the water cultures by adding small amounts of magnesium sulphate to the deficiency cultures (cf. fig. 3).

A great number of soil samples from fields with diseased crops were tested with *Aspergillus niger* on the available magnesium. Figs. 4 and

[1]) It must be emphasized that detrimental effects of low pH-values in media well provided with magnesium may be obtained. These injuries which will be discussed in a subsequent paper are not identical with those of the "Hooghalen disease".

5 contain the results of these investigations. Fig. 4 deals with samples from experimental plots and other fields of well-known properties; the soils of fig. 5 were received from farmers and agricultural advisers in different parts of Holland. The samples from one field are always placed together in the graphs.



Figs. 4 and 5. Available Magnesium in γ per 3 grams of soil.

It is revealed by these figures that soils bearing healthy plants have an available magnesium content being nearly always 100 γ or more in 3 grams of soil. The pH-value of these soils generally lies above 5. Soils with diseased plants, on the contrary, contain very little available magnesium. The pH-values of these soils are mostly between 4.2—5. Evidence that the bad growth of *Aspergillus niger* is really due to the low magnesium content, and not to the acid reaction of the soil added, is given by the fact that mixing the soil samples with 0.5 gram of magnesium-free calcium carbonate did not change the growth of the fungus; the addition of 500 γ of magnesium, however, brought about a normal growth.

The low content of available magnesium in acid soils is not due to the absence of magnesium in these soils. This could be proved by calcinating the samples during 2—3 hours at 500°C., and adding the ash to the *Aspergillus* flasks. It appeared that in many cases the amount

of available magnesium in the ash was more than 20 times higher than that in the soil.

The absence of available magnesium in acid soils is most probably due to the leaching of the Mg-ions under the influence of the low pH. This could be proved by the following experiment. To a slightly acid sandy soil with a high content of available magnesium, increasing amounts of dilute sulfuric acid were added, in order to bring the pH down to 4.1, 3.9, 3.6, 2.8, and 2.2 respectively. After 2 days the soils were dried and 6 days later 3-gram portions were added to *Aspergillus* flasks. A fixation of magnesium in the acid soils, as reported by GEHRING, could not be observed. The same acid soil, however, leached on a filter with distilled water till no more SO_4-ions could be detected in the filtrate, had lost practically all of the available magnesium.

6. EFFECT OF NEUTRALIZATION.

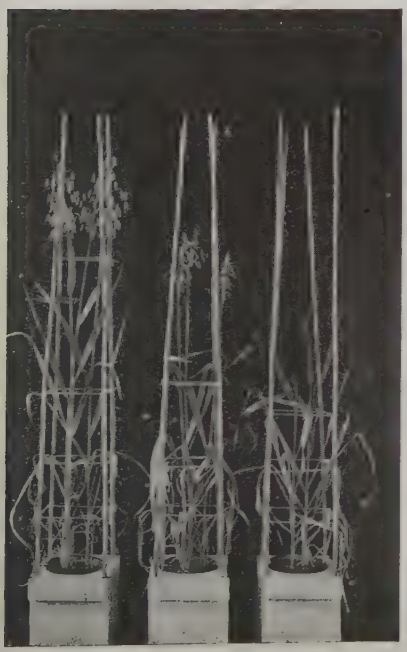


Fig. 6. White oats growing in acid soil. Right: untreated soil, center: addition of $CaCO_3$, and left: addition of $MgSO_4$.

Soils on which the plants are suffering from the "Hooghalen disease" generally may be improved by adding neutralizing substances such as $CaCO_3$, CaO or by fertilizing with $NaNO_3$. It is not yet known how this favourable effect of the neutralization is to be explained. Partly it is due to the magnesium content of the lime used (in two samples of $CaCO_3$ magnesium contents of 0.2 and 0.5 per cent were found). Very pure $CaCO_3$, however, although not increasing the amount of available magnesium, also gave a better growth of the plants (fig. 6, Table II).

Evidently the improved plant growth in the limed soils must be ascribed to an increased assimilation of magnesium which is due to the better developed root system or to an easier absorption of Mg-ions by the root cells in the less acid media.

Table II.

Effect of $CaCO_3$ and $MgSO_4$ on the available magnesium in soils and on the yield and magnesium content of oat plants.

Samples	Available magnesium per 3 g of soil in γ (after cropping)	seed		straw	
		dry matter in g [1])	Mg in mg [1])	dry matter in g [1])	Mg in mg [1])
acid sandy soil A	25—50	0	0	2.66	0.35
with $CaCO_3$	25	3.41	1.66	5.31	0.50
with $MgSO_4$	300	6.01	7.35	5.60	6.79
acid sandy soil B	25—50	1.08	0.62	4.66	0.44
with $CaCO_3$	25	4.75	3.77	7.31	0.52
with $MgSO_4$	300	7.81	9.81	7.17	7.45

[1]) average values from duplicate samples.

7. MICROBIOLOGICAL DETERMINATION OF SMALL AMOUNTS OF MOLYBDENUM.

Recent investigations from ARNON and STOUT (1) have shown the indispensability of molybdenum for the growth of tomato plants. From previous publications it appears that under certain conditions *Azotobacter chroococcum* (2) and *Aspergillus niger* (7, 8, 11) do not thrive well without the presence of small amounts of this element in the nutrient media. Although it is unknown whether molybdenum deficiency of higher green plants is found in natural soils, it may be of some importance to determine the available molybdenum in these media. For these investigations *Azotobacter chroococcum* (VAN NIEL (9)) as well as *Aspergillus niger* may be used.

Summary.

A description is given of some microbiological tests for the determination of plant-available copper, magnesium and molybdenum in soils. In these investigations *Aspergillus niger* and in a few cases *Azotobacter chroococcum* and *Bact. prodigiosum* were used. From the figures obtained it is revealed that soils on which the plants are suffering from the so-called "reclamation disease", have a much lower available copper content than those producing healthy crops. Soils on which the plants show the so-called "Hooghalen disease", have a very low

content of available magnesium. From these results and from experiments with cereals it is concluded that the "reclamation disease" is caused by a deficiency of plant-available copper and that a deficiency of available magnesium is the chief cause of the "Hooghalen disease".

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(From the Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa, U.S.A.)

DISSIMILATION OF CITRIC ACID BY *STREPTOCOCCUS PARACITROVORUS* [1])

by

C. R. BREWER and C. H. WERKMAN.

(Received January 12, 1940).

1. INTRODUCTION.

The action of the citric acid-fermenting, aroma streptococci in the production of desirable flavor and aroma in butter cultures has been investigated by many workers, both in the United States and Europe. See HAMMER (5) for a review of literature dealing with the aroma-forming bacteria of dairy cultures through 1937.

Since HAMMER's review, VAN BEYNUM and PETTE (1,2) have published the results of a comprehensive study of the decomposition of citric acid by butter aroma bacteria. The Dutch workers found that citric acid is decomposed to acetic and carbonic acids, ethyl alcohol, 2,3-butylene glycol, acetylmethylcarbinol and diacetyl. The last 3 compounds are formed only in acid medium. A scheme of fermentation including pyruvic acid as a hypothetical intermediate was presented.

As a result of the investigations carried out in several laboratories, it is now commonly accepted that these organisms ferment the naturally occurring citrate of milk to several products, among which, volatile acids and the three 4-carbon neutral compounds, diacetyl, acetylmethylcarbinol and 2,3-butylene glycol are of most importance from the flavor standpoint.

Nearly all such investigations have been carried out in milk, which possesses the obvious advantage of a natural growth medium but leads to many difficulties in chemical analysis. In addition to the analytical inaccuracies resulting from the physical and chemical complexity of

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[1]) Journal paper No. **J711** of the Iowa Agricultural Experiment Station, Project 451.

milk, it is practically impossible to determine the physiological behavior of organisms with respect to any specified substrate, because of the simultaneous action on other substrates naturally present. For example, *Streptococcus paracitrovorus* attacks the citrate of milk but simultaneously ferments the lactose also present. Obviously, chemical analysis of the fermented milk cannot accurately reveal the source of each final product. It thus becomes necessary to choose other less natural but more specific, methods of investigation.

The present communication reports the results of studies on the dissimilation of citric acid by *Streptococcus paracitrovorus*, and the effect of sugar on this fermentation. The chemical methods and fermentation techniques were those employed in previous investigations of the bacterial dissimilation of citric acid (Brewer and Werkman (3,4)).

2. DISSIMILATION OF CITRIC ACID.

Preliminary experiments with yeast extract media showed that although satisfactory growth was obtained when glucose or lactose was added, no growth occurred in the same basal medium alone or with added sodium citrate as a carbon source. Media containing both citrate and sugar gave better growth than sugar alone. The necessity of sugar in media for growth of aroma bacteria was first pointed out by Knudsen and Sørensen in 1929 (7).

Quantitative determination of the products of the glucose fermentation showed a relatively simple distribution of products (Table I). One mole of glucose is apparently converted to approximately one mole each of lactic acid, ethyl alcohol and carbon dioxide. The first line of the table presents the dissimilation of glucose plus citrate. More glucose was fermented in the presence of citrate than in its absence, although in neither case was the entire 50 mM per liter originally present converted. The 40 mM of citrate per liter were entirely broken down.

Large quantities of acetic acid accumulated during the dissimilation of the combined substrate, but none resulted from the fermentation of glucose. This behavior is in contrast to the work of Hucker and Pederson (6), who found volatile acid in fermentations of glucose by *Leuconostoc*. A small amount of 2,3-butylene glycol was formed from the combined substrate but none from glucose alone. Other differences are: a reduction in the yield of ethyl alcohol and an increase in CO_2 and lactic acid in the medium containing both sugar and citrate.

The third line of Table I, calculated by subtracting the products

Table I.

Anaerobic dissimilation of glucose plus citric acid by *Streptococcus paracitrovorus*.

Products expressed as mM per 100 mM glucose fermented. Duration of experiment 9 days.
Substrates: 0.05 M glucose, 0.04 M citrate.

Glucose fermented/l	Citrate fermented/l	Citrate fermented/ 100 mM glucose	Final pH	CO_2	Formic acid	Acetic acid	Ethyl alcohol	2,3-Butylene glycol	Lactic acid	Carbon recovery %	Redox index
86.8	80.0	92.0	5.5	204.2	5.8	147.0	41.4	5.4	168.4	96.5	1.10 [1]
73.2	0	0	4.2	105.0	3.7	0	94.3	0	98.0	98.6	1.13 [1]
Products from citrate		92.2		99.2	2.1	147.0	–52.9	5.4	70.4	94.4	1.05
Products/100 mM citrate				107.6	2.3	159.4	–57.4	5.9	76.4	94.4	1.05

[1]) Redox balances are high partly because of failure to determine hydrogen. Acetylmethylcarbinol and succinic acid were not found.

of the fermentation of glucose alone from those of the fermentation of glucose plus citrate, represents approximately the dissimilation of citrate under the conditions employed. The fourth line of the table presents the data of the third line calculated to a basis of 100 mM of citrate fermented, for comparison with later tables. Inspection of these data shows that the principal products were carbon dioxide, acetic and lactic acids, with a substantial production of 2,3-butylene glycol. The function of the glucose in the mixed substrate may be to provide a hydrogen donator; *e.g.*, ethyl alcohol, essential to the citrate metabolism of the organism.

Since microrespirometric experiments (see below) demonstrated that small quantities of glucose catalyzed the decomposition of citrate by *S.paracitrovorus*, experiments for chemical analysis were set up using a medium containing a high ratio of citrate to glucose. Table II presents the results of an experiment in which 0.05 M citrate and 0.005 M glucose were used. The glucose was entirely fermented and nearly 80% of the citrate was broken down. The products agree in general with those shown in Table I. Under the conditions of the experiment shown in Table II, considerable quantities of pyruvic acid accumulated. Its identity was established by preparation of the 2,4-dinitrophenylhydrazone, which melted at 213° C. uncorrected, both when pure and when mixed with authentic pyruvic 2,4-dinitrophenylhydrazone.

The yields of acetylmethylcarbinol and 2,3-butylene glycol were larger than in the previous experiment. The high carbon recovery may be due to interference by pyruvic acid in the volatile acid determination. The bottom line of Table II represents approximately the dissimilation of citric acid.

Several attempts were made to determine the products of the dissimilation of citrate alone, both by growing cells and non-proliferating cell suspensions, but insufficient conversion of citrate to allow of accurate conclusions to be drawn was obtained.

The accumulation of pyruvic acid under certain conditions, suggests that it is an intermediate in the fermentation. Accordingly, the dissimilation of pyruvate was investigated. Table III presents the results of one experiment. The products obtained are similar to those formed from citrate plus glucose.

After the foregoing experiments were performed, the work of VAN BEYNUM and PETTE (1) appeared, showing similar results. The Dutch workers postulated that pyruvic acid was an intermediate compound, and studied its dissimilation but did not isolate it from their fermentations.

Table II.

Anaerobic dissimilation of glucose plus citrate by *Streptococcus paracitrovorus*.

Products expressed as mM per 100 mM citrate fermented. Duration of experiment 7 days.
Substrates: 0.05 M citrate, 0.005 M glucose.

Glucose fermented/l	Citrate fermented/l	H_2	CO_2	Formic acid	Acetic acid	Ethyl alcohol	Acetyl-methyl-carbinol	2,3-Butylene glycol	Pyruvic acid	Lactic acid	Carbon recovery %	Redox index
5.0	38.9	1.8	158.9	0	107.3	15.3	9.7	2.7	38.0	62.1	111.3	0.99
Products from glucose on basis of Table I			13.5	0.5		12.2				12.6		
Net products from citrate		1.8	145.4	–0.5	107.3	3.1	9.7	2.7	38.0	49.5	113.0	0.98

Table III.

Anaerobic dissimilation of pyruvic acid by *Streptococcus paracitrovorus*.

Products expressed as mM per 100 mM pyruvate fermented. Duration of experiment 7 days. Substrate: 0.092 M pyruvate.

Pyruvate fermented	H_2	CO_2	Formic acid	Acetic acid	Ethyl alcohol	Acetylmethyl-carbinol	2,3-Butylene glycol	Lactic acid	Carbon recovery %	Redox index
29.0	3.4	60.8	26.2	42.4	3.7	3.8	7.4	31.4	106.1	1.05

Other experiments (not shown) with lactose and galactose have shown that these sugars are dissimilated by *S.paracitrovorus* to products similar to those from glucose. Glucose is most rapidly and galactose least rapidly fermented of the three sugars.

3. CATALYTIC EFFECT OF GLUCOSE ON DISSIMILATION OF CITRIC ACID.

The Barcroft-Warburg respirometer technique was used as a second method of investigation of the action of combined substrates. The bacteria were grown in glucose-citrate-yeast extract media, harvested by centrifuging, washed once with distilled water and suspended in 0.1 molar phosphate buffer of the desired pH. Usually 2% of cell paste was used.

Figure 1 illustrates the marked effect of combined substrates on the gas production of *S.paracitrovorus*, S-9, at a pH of 6.3. Although the gas production from either citrate, glucose or lactose alone was rather low, combination of citrate with either glucose or lactose resulted in a rapid and large gas evolution.

KREBS and JOHNSON (8) reported a catalytic effect of citrate on oxidative muscle glucolysis. They found that 0.006 molar arsenite inhibited catalysis. To determine whether some of the reactions of the KREBS' citrate cycle apply to the *S.paracitrovorus* dissimilation, arsenite was added to the substrate in the respirometer side cups. The curves shown indicate that this inhibitor had no significant effect. Since the catalytic effect found with *S.paracitrovorus* occurs anaerobically and is insensitive to arsenite, it seems that the KREBS system does not operate.

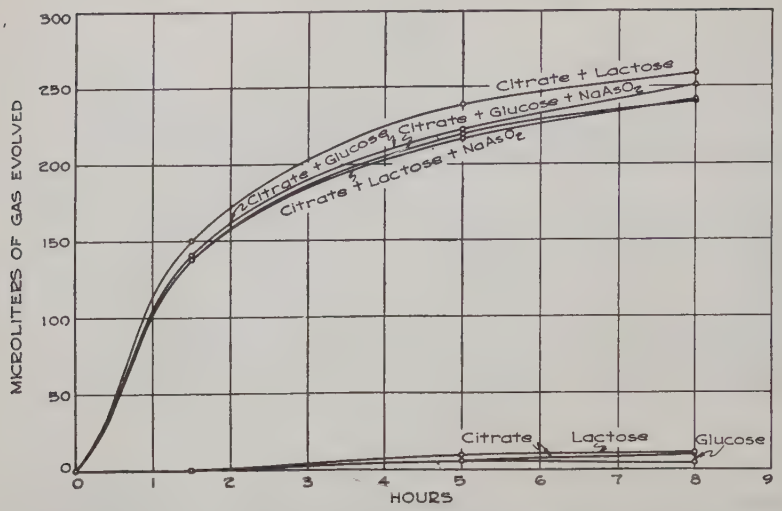

Fig. 1. Effect of combination of citric acid and glucose or lactose and of arsenite on the anaerobic metabolism of *Streptococcus paracitrovorus* S-9 at pH 6.3. 0.2% substrates used.

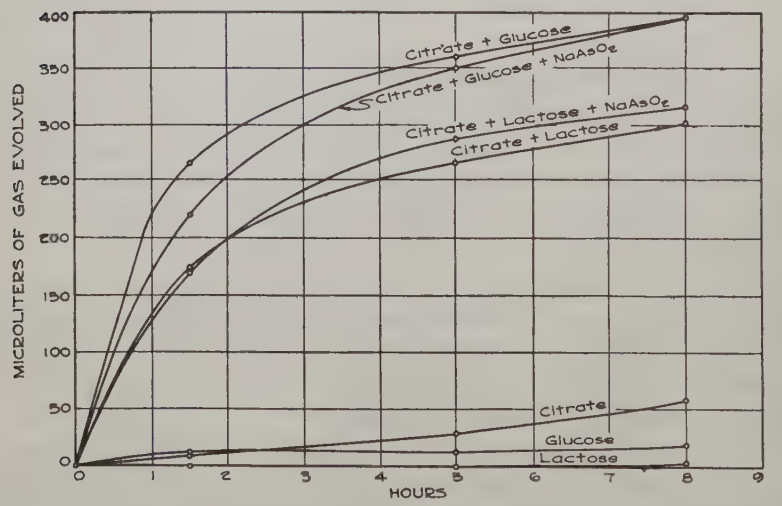

Fig. 2. Effect of combination of citric acid and glucose or lactose and of arsenite on the anaerobic metabolism of *Streptococcus paracitrovorus* S-9 at pH 5.0. 0.2% substrates used.

Figure 2 shows the results of a similar experiment carried out at a lower pH which should more closely approximate the acidity of a butter culture. The same type of catalytic action occurred but was even

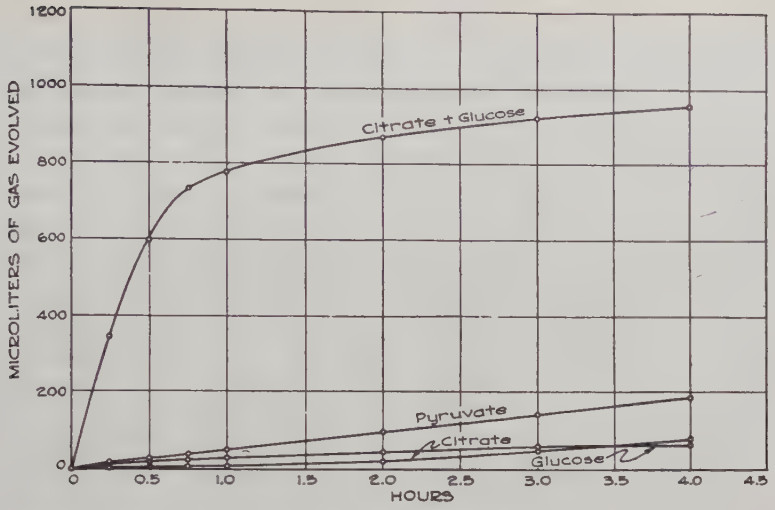

Fig. 3. Effect of combination of citric acid and glucose on the anaerobic metabolism of *Streptococcus paracitrovorus* MU29 at pH 6.3. 0.2% substrates used.

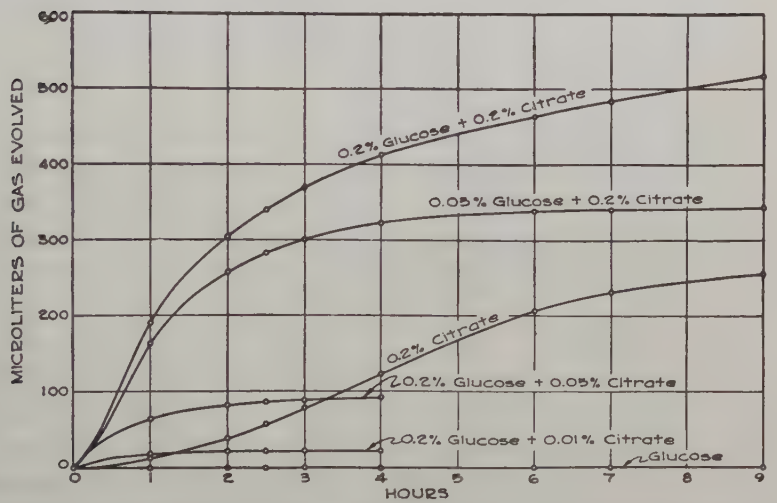

Fig. 4. Comparative catalytic effects of low concentrations of citric acid and glucose on the anaerobic metabolism of *Streptococcus paracitrovorus* S-9 at pH 6.3.

more rapid than at the higher pH, as evidenced by the steep slope of the curve in the first 2 hours of the experiment.

Figure 3 illustrates the data obtained with a different strain of the

same species. The same catalytic effect is apparent. The activity on the combined substrate was much greater than on pyruvate.

Similar catalytic effects were obtained in experiments carried out in the presence of air. Although ample oxygen was present, little was consumed as measured manometrically.

It is obvious that the bacteria metabolize the combined substrates of citrate and glucose much more rapidly than either alone. By varying the relative concentrations of the substrates, it should be possible to determine whether glucose or citrate exerts the catalytic effect. Figure 4 shows curves obtained from such an experiment. During the first 2 hours, when evolution was most rapid, the gas produced from citrate alone and glucose alone was small as usual. During the same period, the curves representing 0.2% citrate with 0.2% glucose and 0.05% glucose rise rapidly together while those of 0.2% glucose plus 0.05% citrate and 0.01% citrate rise more slowly to maxima evidently dependent on the citrate concentrations. Thus, it would seem that citrate is the source of most of the gas and that glucose is the catalytic agent.

Further manometric experiments were carried out in search of other compounds catalyzing citric acid decomposition in a manner analogous to glucose and lactose. The following compounds and extracts were used: fumarate, succinate, lactate, pyruvate, formate, hexosediphosphate, galactose, arabinose, mannitol, ascorbic acid, riboflavin, nicotinic acid, acetylmethylcarbinol, ethyl alcohol, thiosulfate, ether extract of yeast extract, boiled yeast juice and the medium from which the bacteria had been centrifuged, both boiled and unboiled. None of these substances successfully replaced glucose as a catalyst, although slight stimulations were noted.

4. DISCUSSION.

These data indicate that *S.paracitrovorus* possesses an enzyme system which attacks a combined substrate of citrate and lactose or glucose with greater rapidity than any one of them singly. Since milk contains such a combined substrate, it is logical to assume that the bacteria act on both citrate and lactose. Thus, it becomes necessary to deal with the dissimilation of citrate plus lactose in studying the physiology of butter culture bacteria rather than with citrate alone.

Several theories may be offered to explain the catalytic effect of sugar on citrate dissimilation. There may be a direct reaction between citrate and sugar. The sugar may act as a hydrogen donator or acceptor. It may serve as a basis for synthesis of some complex activator or coen-

zyme. The fact, that citrate alone is slowly broken down after a lag phase of 4 to 6 hours, indicates that the coenzyme or activator theory may be correct. Since the addition of several other hydrogen donators and acceptors did not produce a catalysis comparable to that of glucose, it is unlikely that the catalytic activity of glucose is due entirely to the donation of hydrogen to citrate.

The failure of arsenite to inhibit the activity of *S.paracitrovorus* on citrate plus glucose indicates that the mechanism of dissimilation by this organism is not like that of *Aerobacter indologenes* which was practically completely inhibited by arsenite (BREWER and WERKMAN (4)). Another important difference between the glucose-catalyzed fermentation of citrate by the butter culture streptococci and the normal citrate metabolism of coli-aerogenes bacteria is the complete absence of succinic acid from media fermented by the streptococci, whereas succinate is formed in relatively large quantities by coli-aerogenes bacteria.

Manometric experiments with *A.indologenes* showed that the addition of small quantities of glucose to citrate dissimilations has no catalytic effect. *Aerobacter* and *Citrobacter* ferment citrate rapidly in the absence of sugar, therefore, it is not surprising that no catalysis could be demonstrated.

The dissimilation of glucose by *S.paracitrovorus* appears to be a combination of the lactic and alcoholic fermentations. The isolation of phosphoglyceric acid by STONE and WERKMAN (9) from *S.paracitrovorus* suspensions suggests that the EMBDEN-MEYERHOF-PARNAS scheme of glucolysis applies to this organism. If this is true, pyruvic acid will be the principal intermediate from which lactic acid will be formed by direct reduction. Ethyl alcohol will be produced by reduction of the acetaldehyde formed by decarboxylation of pyruvic acid.

The dissimilation of citric acid by *S.paracitrovorus* probably proceeds in a manner similar to that postulated for *A.indologenes*. The citric acid will be split into acetic and oxaloacetic acids. The latter will decarboxylate to form pyruvic acid which will undergo the generally postulated reactions for the formation of lactic acid, acetaldehyde and CO_2, with the condensation of the aldehyde to form acetylmethylcarbinol and 2,3-butylene glycol. The apparent connection between the fermentations of glucose and citrate by *S.paracitrovorus* may be the oxidation of some reduced metabolite of the former, probably acetaldehyde, coupled with the reduction of the pyruvic acid formed from

citrate to lactic acid or through acetylmethylcarbinol to 2,3-butylene glycol.

Summary and Conclusions.

S.paracitrovorus does not readily dissimilate citric acid in the absence of sugar but does attack citric acid relatively vigorously in the presence of small quantities of glucose or lactose. The effect of glucose and lactose in initiating the dissimilation of citric acid is catalytic.

The sugars which act catalytically are themselves fermented to approximately equimolar quantities of carbon dioxide, ethyl alcohol and lactic acid. The dissimilation of a combined substrate of citrate and glucose forms, in addition, acetic acid, acetylmethylcarbinol, 2,3-butylene glycol and under certain conditions, pyruvic acid which acts as an intermediate compound. Pyruvate is dissimilated to products similar to those from a fermentation of citrate plus glucose.

The reactions of KREBS' citric acid cycle apparently do not apply to the dissimilation of citric acid by *S.paracitrovorus* because the fermentation of citric acid proceeds anaerobically, consumes little oxygen aerobically and is not inhibited by arsenite.

Inasmuch as milk contains lactose, the fermentation of citric acid in milk by *S.paracitrovorus* may be catalyzed as shown in these studies.

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(From the Laboratory of Microbiology, Technical University, Delft.)

THE SUITABILITY OF DISACCHARIDES AS RESPIRATION AND ASSIMILATION SUBSTRATES FOR YEASTS WHICH DO NOT FERMENT THESE SUGARS

by

A. J. KLUYVER and M. TH. J. CUSTERS.

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1. INTRODUCTION.

In the older literature on the physiological properties of yeast species it is not at all uncommon to find that a discrimination is made between the sugars which can be fermented by a given species and those which are able to act as a substrate for the assimilation of the yeast. We may cite here by way of example the publications of ROSE (27), LINDNER and SAITO (21), and KLUYVER (16). Characteristic of the viewpoint exposed in these earlier publications is that their authors accept without further discussion that certain sugars are suitable as a sole source of carbon, although these sugars — in contradistinction to hexoses like glucose [1]) — are not fermented. At first sight this attitude is easily understood, since there is not the slightest doubt that certain yeast species can indeed use fully unfermentable compounds like lactates, glycerol and various hexitols as assimilation substrates, at least in so far as an ample supply of oxygen is provided. Under these conditions it seems qui e acceptable that the same situation also applies to the case of unfermentable sugars, and there are indeed several reports according to which under aerobic conditions the unfermentable pentoses are indeed attacked by certain yeasts (26, 19).

In the light of our present knowledge of metabolism the question is, however, quite different as far as unfermentable disaccharides (or more generally: polysaccharides) are concerned. For in this case the

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[1]) It may be remarked here once for all that yeasts which do not ferment glucose, and therefore have no fermentative power at all, are excluded from the present discussion.

lack of fermentability seems to offer conclusive proof for the view that the cells in question lack the ability to bring about a hydrolysis of these sugars. Accepting this, one is forced to conclude that the organism is able to synthesize its numerous different cell compounds from the unsplit disaccharide, a view which seems utterly absurd. Moreover, one has to realize that assimilation cannot proceed without the simultaneous occurrence of an energy yielding process, which in the case of an unfermentable compound inevitably implies that this compound acts partly as a substrate of the respiration process.

Now it is also quite difficult to conceive that a disaccharide will undergo oxidation without preceding hydrolysis. In this case one might expect that under certain conditions incomplete oxidation products containing more than 6 carbon atoms and still having the characteristic oxygen bridge of the disaccharide would have been found, and this is contrary to all experience. Such a „direct" oxidation of a disaccharide — *i.e.*, without previous hydrolysis — is further quite incompatible with the unitarian theory of respiration and fermentation.

It was especially with a view to the latter consideration that some years ago the senior author, together with HOOGERHEIDE (17), has examined more closely the statement of TRAUTWEIN and WEIGAND (32) that certain non-maltose-fermenting yeast species — *Saccharomyces Marxianus* Hansen and *Saccharomyces exiguus* (Reess) Hansen — were able to use maltose as a respiration substrate. It was then shown that this statement could not be upheld; all experimental data being in favour of the view that the increase in respiration observed on addition of maltose was due to the presence of impurities in the commercial preparations of the sugar.

In consequence hereof it was thought at that time that the question of respiration of unfermentable disaccharides, and therefore also that of the assimilation of these sugars, was dead and buried.

However, in the course of an investigation on the properties of various yeast species belonging to the genus *Brettanomyces* the junior author came across a clear case of the assimilability of saccharose by a non-saccharose-fermenting strain. This strain has since been described under the name of *Brettanomyces anomalus* Custers (6).

On considering the problem involved in this observation our attention has been drawn to several statements in publications of other workers in the field of yeast taxonomy, in which also unmistakably positive results were reported regarding the assimilability of unfermentable disaccharides. We refer here especially to the papers of

Redaelli (25), of Ciferri and Alfonseca (4), of Ciferri and Redaelli (5) and those of Langeron and Guerra (18), although others could be added. It is true that these authors do not offer any special comment to their results, but nevertheless their findings must be considered as serious arguments against the views put forward by Hoogerheide and the senior author. For although impurities might affect the very sensitive manometric method for the establishment of respiration, they could scarcely be responsible for the obviously considerable proliferation of yeast cells observed by the authors cited.

For this reason we have thought it desirable to reinvestigate the problem outlined above. In the first place we have chosen for further inspection the case of *Mycocandida parakrusei* (Cast.) Langeron et Guerra for which the last mentioned authors report that this organism is able to assimilate saccharose and maltose, although these sugars are not fermented. At a time that our investigation had already made considerable progress, and had already been extended to a few other species, another example of the discussed abnormal behaviour of a yeast species was kindly brought to our notice by Hoeke. This investigator showed us some of his observations which left no doubt that *Torulopsis dattila* (Kluyver) Lodder, a yeast which is generally accepted to be unable to ferment maltose, is successful in attacking this sugar, if the organism is grown under aerobic conditions in a medium in which maltose is the only carbohydrate present [1]). This result was especially striking, because since 1914 the said yeast species has frequently been employed in the senior author's biochemical method for sugar determination, on the basis of its inability to attack maltose under anaerobic conditions.

Hoeke's results induced us to give further extension to our investigation, and we decided to include also an examination of some other apparently related statements in literature which had puzzled us for a long time.

In the famous chapter entitled „Théorie physiologique de la fermentation" of his „Etudes sur la bière" Pasteur (23) describes an experiment in which he inoculates yeast in a medium consisting of yeast water with 2.5% lactose. He then reports further that, although the yeast was quite unable to ferment this sugar, a quite satisfactory yeast crop was obtained after three months.

Pasteur attached great importance to this result. He had already shown that under certain conditions a mould (*Mucor* species) could

[1]) The results have since been published; cf. (9).

live and act like a yeast, *i.e.*, could give rise to fermentation. From the experiment reported he drew the conclusion that conversely a yeast could behave like a mould, *i.e.*, could develop at the expense of an unfermentable sugar. However, it must be remarked that no direct proof was offered for this. The possibility that the yeast had been growing merely at the expense of the constituents of the yeast water does not seem to have occurred to PASTEUR.

It seemed worthwhile to repeat this experiment with ordinary strains of baker's yeast and brewery yeast, all of which are devoid of the ability to ferment lactose.

A further inducement hereto was found in a more recent publication of HOFMANN (10) which author claims the startling result of having demonstrated the presence of lactase in bottom yeasts. The experimental proof for this assertion is open to serious criticism for which we refer to Chapter 6 of this paper. On the other hand HOFMANN's findings seemed to find support in PASTEUR's observation described above.

Also a later publication of HOFMANN (11) asked for a reinvestigation. Herein this author describes that he was able to demonstrate the presence of saccharase in *Schizosaccharomyces octosporus* Beijerinck. This fact seemed incompatible with BEIJERINCK's emphatic statement that this yeast species is unable to ferment saccharose, although glucose and maltose are vigorously fermented.

On the ground of the foregoing exposé it seemed desirable to make a more or less systematic study of the ability of various yeast species to attack under different conditions disaccharides which according to the data in literature are not fermented by the species in question.

Such a study should, of course, embrace both an investigation of the suitability of the disaccharide as a respiration substrate, and its assimilability in growth experiments.

Taking into account the various reports mentioned above, we have made a careful selection of yeasts in such a way that a series of species was obtained for which the discussed anomaly had been reported. In addition a few other species were included in the investigation, because it seemed desirable to ascertain their behaviour in the mentioned respect in connection with their possible applications in biochemical sugar analysis. With a view to the demands of the biochemical method for sugar determination special attention was given to those rare species which share with *Schizosaccharomyces octosporus* the property of fermenting maltose, but not saccharose.

All yeast strains were obtained from the collection of the Yeast Division of the „Centraalbureau voor Schimmelcultures".

We shall now give here a survey of these species, together with a brief indication of the reasons for their selection.

A. Non-maltose-fermenting yeast species.

1. *Brettanomyces anomalus* Custers. Although this strain had especially attracted our attention by its property of giving a positive auxanogram with saccharose, we have also studied its behaviour towards maltose, because preliminary experiments roused our suspicion that it would be able to assimilate maltose too.
2. *Mycocandida parakrusei* (Cast.) Langeron et Guerra. Reported by the latter authors to give a positive auxanogram with maltose.
3. *Zygosaccharomyces Marxianus* (Hansen) Guilliermond et Negroni (Syn.: *Saccharomyces Marxianus* Hansen). Claimed by Trautwein and Weigand to use maltose as a respiration substrate, a view which, however, has been combated by Kluyver and Hoogerheide.
4. *Saccharomyces exiguus* (Reess) Hansen. Same argument as under 2.
5. *Torulopsis dattila* (Kluyver) Lodder. Reported by Hoeke (9) to attack maltose under aerobic conditions.
6. *Torulopsis utilis* (Henneberg) Lodder. Provisionally recommended by Hoeke as suitable for biochemical sugar determinations in which maltose should be preserved.
7. *Saccharomycodes Ludwigii* Hansen. A yeast species reported by Gottschalk (8) to be able to hydrolize starch.
8. *Saccharomyces fragilis* Jörgensen. A species for which the same behaviour as mentioned under 7 has been established by Mr. F. Smits van Waesberghe.
9. *Torula cremoris* Hammer et Cordes. Recommended by van Voorst (33) as suitable for biochemical sugar determinations in which maltose should be preserved.
10. *Torula monosa* Kluyver. This species was included into the investigation in order to test its applicability for biochemical sugar determinations under aerobic conditions. The species ferments neither saccharose, nor maltose.

B. Non-lactose-fermenting yeast species.

1. *Blastodendrion intermedium* Ciferri et Ashford. Langeron and

Guerra report that this species gives a positive auxanogram with lactose.

2. *Saccharomyces carlsbergensis* Hansen. A bottom yeast of the type used by Hofmann (l.c.) in his experiments on the occurrence of lactase in brewery yeast.
3. *Saccharomyces cerevisiae* Hansen. A top yeast of a type as may well have been used by Pasteur in his experiment referred to above.

C. Non-saccharose-fermenting yeast species.

1. *Brettanomyces anomalus* Custers. Reported by the author to give a positive auxanogram with saccharose.
2. *Mycocandida parakrusei* (Cast.) Langeron et Guerra. Reported by the latter authors to give a positive auxanogram with saccharose.
3. *Mycotorula albicans* (Robin) Langeron et Talice (strain 493, McKinnon). Langeron and Guerra report that this species does not ferment saccharose, but gives a positive auxanogram with this sugar.
4. A second strain of the preceding species (strain Levy). Argument given under 3.
5. A third strain of the preceding species, but labeled *Blastodendrion erectum* Langeron et Talice (strain 417a of the collection of Sabouraud). Argument given under 3.
6. *Schizosaccharomyces octosporus* Beijerinck. In spite of Beijerinck's claim that this species does not ferment saccharose, it is reported by Hofmann (l.c.) to contain saccharase.
7. *Saccharomyces italicus* Castelli. A species recently described by Castelli (3), and claimed to have the rare property of fermenting maltose, but not saccharose.
8. *Torula* species Claussen. The „Centraalbureau voor Schimmelcultures" originally received this strain from Claussen under the name of *Saccharomyces Pastorianus*. As a result of the investigation by Stelling-Dekker (30) this strain was relabeled. For this strain the same behaviour as described under 7 should hold.
9. *Torula monosa* Kluyver. Argument given under A 10.

2. OUTLINE OF THE INVESTIGATION AND METHODS APPLIED.

a. Fermentation.

For every yeast species it was in the first place carefully tested whether the reports in literature that a certain disaccharide was un-

fermentable could be corroborated. For this purpose it seemed desirable to have recourse to several methods. On the one hand it seemed indicated to apply a routine method as used by the majority of the investigators cited, in order to be able to judge more directly their results. Since, however, these methods all lack sensitivity — as has fairly recently been exposed in the monographs of STELLING-DEKKER (30) and of LODDER (22), to which the reader is referred — it was necessary to give further extension to the investigation. From the discussion given in Chapter 1 it will be clear that it was of the utmost importance to decide whether the ability to attack a disaccharide under aerobic conditions was always accompanied by a weak fermentative power towards the carbohydrate in question.

These considerations are responsible for the circumstance that we in all cases have tested the fermentability of the disaccharide according to each of the four methods following.

1. *Method of the Einhorn fermentation tube.*

The advantages and disadvantages of this routine method for the testing of the fermentative power of a yeast species have fully been discussed by STELLING–DEKKER. Thus nothing needs to be said here regarding this point. It may suffice to remark that the method was applied in the more sensitive modification recommended by the last mentioned author who rightly emphasizes the importance of shaking the liquid at regular intervals in order to attain a good distribution of the yeast cells also in the closed arm of the tube. Inspection as to the presence of gas in the closed arm was made daily; the observation was, if necessary, always extended to 10 days.

2. *Method of the Struyk fermentation flask.*

A few years ago STRUYK (31) has drawn the attention to a special type of fermentation flask, devised by him, which offers several advantages in the testing of the fermentability as compared with the Einhorn tube. Both instruments have in common that they at the same time allow the establishment of gas evolution from the medium, and yet allow free access of the air to the inoculated sugar medium. The latter factor is indispensable in the case of yeasts which as a rule only show very restricted proliferation under strictly anaerobic conditions. The Struyk flask has been constructed in such a way that the yeast cells which originate in the aerobic part of the medium have a tendency to accumulate in the anaerobic part of the flask, whilst any

fermentation gas produced there is collected under the stopper of the flask.

The general experience in this laboratory is that indeed in several cases of weak fermentation positive results in the Struyk flasks have been obtained, although the Einhorn tube failed to indicate fermentation. The observations in the Struyk flask were also extended over 10 days.

3. *Control of the fermentability of a sugar in the quantitative apparatus after van Iterson–Kluyver* (Cf. (15)).

We deemed it desirable to test the fermentability of the sugar also in the said quantitative apparatus. Herein a small volume of the sugar-containing medium is inoculated with a relatively large quantity of the yeast to be tested. In consequence hereof a considerable multiplication of the yeast is unnecessary, and this principle makes it possible to test the fermentative power of the yeast under strictly anaerobic conditions. In case of a restricted fermentability of the sugar the sensitivity of the method can be largely increased by adding to the medium a suitable amount of a sugar which is strongly fermented. In our experiments we always dissolved 2% of the disaccharide to be tested in a yeast water medium which already contained about 2% glucose. Any fermentation of the disaccharide should express itself in an increase of the amount of gas produced, as compared with a blank experiment in which the glucose containing medium without addition of the disaccharide was fermented.

4. *The manometric method of Warburg.*

Undoubtedly by far the most sensitive way of establishing and measuring a fermentation process is the manometric method as developed by WARBURG. This method is too well known to need description. With the aid of this method fermentation can be established under strictly anaerobic conditions. We always studied the fermentation in an atmosphere of nitrogen which had been carefully freed from oxygen by passing it over copper gauze electrically heated at a temperature of about 500° C.

In all cases the yeast cells obtained from malt agar plates were suspended in tap water, centrifuged, washed again with tap water, centrifuged, and these operations repeated once more. The collected cells were suspended in a phosphate buffer pH = 4.5. A suitable amount of the sugar to be tested, equally dissolved in the said buffer solution,

was always added from a side tube after equilibrium in the Warburg-vessel had been attained.

A parallel experiment was always run with glucose as a fermentation substrate, whilst as a rule the fermentation was also tested in the absence of any substrate. The temperature of the thermostat was in all cases 30° ± 0,02° C.

b. Assimilation.

After the yeast species had thus been carefully tested on its fermentative power towards the disaccharide in question, it was investigated whether the organism was able to assimilate the sugar, *i.e.*, whether the organism was able to proliferate in a medium in which the sugar was the main source of carbon.

In order to test this point we thought it desirable to apply two independent methods.

1. *The auxanographic method of Beijerinck.*

This method has recently been introduced into practical yeast taxonomy by Lodder (l.c.), and has since been adopted by several other workers in this field. For the details of the method we refer to the publication of Lodder. It may suffice to state here that a positive result of the experiment is based on a visual judgement of an increase in turbidity in the diffusion field of the sugar as compared with that in the blank spots of the agar plate. Control experiments were always made by bringing also fermentable sugars on the same plate.

In many cases the outcome of the experiment is quite decisive: the field of the sugar to be tested being either equally turbid as the blank spot, or showing a marked increase in cloudiness.

However, in other cases the result may be doubtful, the difference in turbidity being so small that one does not feel certain that the slight increase in turbidity observed guarantees a real attack of the sugar, and is not due to the possible presence of small quantities of impurities in the sugar preparation.

In this connection the following should be realized. The auxanographic method is undoubtedly highly satisfactory in case of a quick assimilation of the compound diffusing into the plate. If, however, this compound is only slowly attacked, and its consumption therefore takes a long time, the compound will gradually be evenly distributed in the whole plate, and this circumstance will, of course, cause no local contrasts in growth to appear. It is for this reason that in certain cases of

evidently very slow assimilation the usual technique was modified in such a way that the sugar was kept in a restricted diffusion field, by removing a semi-circular strip of agar all around the spot where the sugar was afterwards deposed.

Nevertheless we felt the need of another method which would allow a more direct establishment of the consumption of the disaccharide tested.

For this reason we have also investigated the assimilability of the disaccharide in the following way.

2. *Growth experiments in liquid media with quantitative determination of the disappearance of the sugar.*

It will not need any comment that this method is especially suitable to decide whether indeed a given yeast species is able to attack a certain disaccharide. This method moreover offers the advantage that the duration of the experiment can be almost indefinitely prolonged, which is of much value in cases in which the assimilation only proceeds slowly.

It will be clear that this method yields very reliable results, at least if due precautions are taken.

All culture experiments were made in Erlenmeyer flasks of 300 cc. Into each flask an accurately weighed amount of disaccharide ($\pm$ 1.5 g) was brought and the sugar dissolved in 20 cc distilled water. Then the flasks and its contents were sterilized in the autoclave at 110° C. during 20 minutes. After cooling 50 cc of sterile yeast water was added. Hereupon the media were rather heavily inoculated from a malt agar tube culture of the yeast to be investigated. The flasks were incubated at 30° C. As a rule all experiments were made in duplicate; one set of cultures was tested for the disappearance of the sugar after 16 days, the second set after 35 days.

For sake of comparison parallel cultures were run in media in which glucose was substituted for the disaccharide. In all cases the glucose had been completely consumed after 16 days, showing that the culture used for the inoculation was indeed in good condition.

The quantitative determination of the disaccharide was made with the aid of SCHOORL's modification of the FEHLING method (in the case of saccharose after inversion with dilute hydrochloric acid). In order to eliminate the difficulties ensuing from the evaporation of the culture medium during the long run of the experiment, the contents of each flask were quantitatively transported into a volumetric flask of 100 cc which was then filled up to the mark. From the resulting solution 5 or 10 cc were used for the sugar determination.

A slight complication was caused by the fact that the yeast water used always contained a small amount of compounds reducing Fehling's solution. For this reason at the beginning of each set of experiments the reduction of a weighed amount of sugar was determined in a non-inoculated flask containing the usual amount of yeast water, and also in an equal volume of distilled water. The difference between the two determinations proved to equal the reducing value of the same amount yeast water when tested alone.

It seemed of importance to decide whether a slight decrease in reducing power of the disaccharide medium after the yeast had been cultivated in it might only be due to a consumption of the reducing compounds present in the yeast water. For this reason all yeast species investigated were also grown for the same period in Erlenmeyer flasks containing 50 cc yeast water and 20 cc distilled water, but no sugar. It may be remarked here once for all that none of the yeasts investigated had caused any significant decrease of the reducing power in the non-sugar-containing media. Although in special cases a slight decrease was observed the differences were so near to the experimental error of the analytical procedure applied that it was quite justified to neglect them.

The control experiments in the sugar-free media were also useful in another respect, since they gave a good demonstration of the fact that several yeast species show a marked development in these media, obviously at the expense of the various constituents of the yeast water. This result offers a fair warning against the not unusual procedure of various investigators to conclude as to the assimilability of a certain sugar, only on account of a visual establishment of proliferation of the organism in a medium containing, besides the sugar, organic nitrogenous compounds like yeast water, peptone etc., also. It is clear that in such cases the sugar need not be attacked at all.

A further precaution made was that also uninoculated flasks containing the various media tested were incubated for a period of 16 days, in order to determine whether a disappearance of the disaccharide took place by a purely chemical action of the yeast water constituents. This control seemed especially important in the case of the easily hydrolizable saccharose. If an inversion of that disaccharide would take place, a disappearance of this sugar in the inoculated flasks would give no guarantee that the organisms in question had the ability to attack the saccharose.

Finally a very thorough test was made for each flask as to the absence

of contaminating organisms in the medium. Hereto in the first place a microscopical examination was made, but besides this a small quantity of the medium was streaked both on peptone agar and on malt agar plates. These plates were then incubated for 2 days at 30° C. Then the plates were carefully inspected for the presence of colonies of any organism other than the yeast culture used in the experiments. In the rare cases that contamination was established the analytical results were, of course, discarded.

c. Respiration.

In Chapter 1 it has been argued that an assimilation of a compound which is not fermented should always be accompanied by a respiration process in which this compound acts as a substrate of the oxidation. In the case of an assimilable, unfermentable disaccharide this should imply that this compound is subject to a direct oxidation, *i.e.*, an oxidation without previous hydrolysis. Since such a result is difficult to conceive, and moreover conflicting with the prevalent theories of oxidation and fermentation, it seemed of the utmost importance that direct proof should be obtained for the suitability of the disaccharide as a respiration substrate. For this the application of the Warburg manometric method seemed again indicated.

Whilst it is unnecessary to enter into a discussion of this method, it is desirable to make a few remarks regarding the interpretation of the results. It is clear that no conclusions regarding the suitability of a compound as a respiration substrate can be drawn without simultaneous determination of the endogenous respiration, *i.e.*, the respiration without addition of a substrate. From the results obtained by Kluyver and Hoogerheide (l.c.) it is further clear that it is inadmissible to interpret any increase in oxygen consumption due to the addition of sugar to a cell suspension as a proof that this sugar is indeed the substrate of the oxidation. The great sensitivity of the manometric method is responsible for the fact that already small amounts of impurities present in the sugar preparation lead to a quite noticeable oxygen consumption. Although the sugar preparations used were the purest available [1]), this disturbing effect could not always be avoided. As a rule it will, however, be possible to discriminate between a „true respiration of the sugar" and a respiration caused by accompanying

[1]) In all our experiments — on fermentation, assimilation and respiration — use was made of „Maltose C.P." and „Lactose C.P." of the Pfanstiehl Chemical Co., and of „Saccharose puriss" of Merck.

impurities by extending the experiment over a longer period of time. If the respiration is due to impurities a decrease of the oxygen consumption with time may be expected; if the sugar itself is attacked a constant respiration rate will be observable at least over periods extending to 4 or 6 hours.

In all our experiments due attention was given to the foregoing considerations.

It may be added that for the sake of comparison always control experiments were made with glucose as a respiration substrate.

3. RESULTS OBTAINED WITH THE NON-MALTOSE-FERMENTING YEAST SPECIES.

In the following an account will be given of the behaviour of the various yeast species enumerated in the list given in Chapter 1 under A.

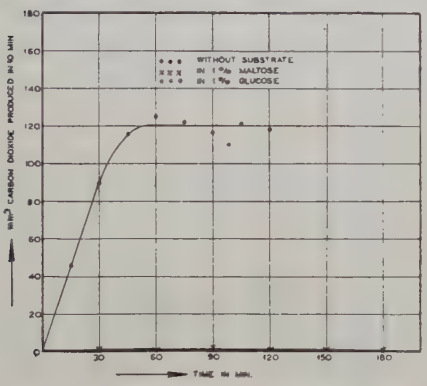

Fig. 1. *Torulopsis dattila* — Anaerobic carbon dioxide production in glucose, in maltose, and in the absence of substrate.

a. Fermentation.

It may suffice to state here that for all the species investigated all tests on their ability to ferment maltose, indeed, yielded completely negative results.

By way of example we give in Fig. 1 a graphic representation of the results obtained in the case of *Torulopsis dattila*, in which figure for the sake of comparison also a fermentation experiment with glucose as a substrate is reproduced.

b. Assimilation.

The qualitative test with the aid of the auxanographic method for the assimilability of maltose gave clearly positive results with *Torulopsis dattila*, *Torulopsis utilis* and *Mycocandida parakrusei*; all other species were negative in this respect.

In the case of the three species mentioned, we have therefore a first confirmation of the results of the earlier investigators regarding the assimilability of a non-fermentable disaccharide.

The growth experiments with quantitative determination of the maltose gave a definite proof for this noteworthy phenomenon, as may be seen from Table I.

Table I.

Maltose consumption in growth experiments with non-maltose-fermenting yeast species.

The initial reducing power of the yeast water present in the culture media was equivalent to 27 mg maltose, the initial reducing power of the maltose-containing media was equivalent to 1470 mg maltose; each culture flask, therefore, initially contained 1443 mg maltose.

Yeast species	After 16 days incubation at 30°C.				After 35 days incubation at 30°C.			
	Reducing power [1]) of the medium	Maltose remaining in medium	Maltose consumed in mg	Maltose consumed in % of initial amount	Reducing power of the medium	Maltose remaining in medium	Maltose consumed in mg	Maltose consumed in % of initial amount
Brettanomyces anomalus	1450	1423	20	1.4	1257	1230	213	14.8
Mycocandida parakrusei	1358	1331	112	7.7	1183	1156	287	19.9
Zygosaccharomyces Marxianus . .	1464	1437	6	0.4	1416	1389	54	3.7
Saccharomyces exiguus	—	—	—	—	1404	1377	66	4.6
Torulopsis dattila	856	829	614	42.6	647	620	823	57.0
Torulopsis utilis	1434	1407	36	2.5	1320	1293	150	10.4
Saccharomycodes Ludwigii . . .	1444	1417	26	1.8	1402	1375	68	4.7
Saccharomyces fragilis	1438	1411	32	2.2	1452	1425	18	1.2
Torula cremoris	1464	1437	6	0.4	1462	1435	8	0.6
Torula monosa	—	—	—	—	1454	1427	16	1.1
Control with maltose-fermenting yeast: *Saccharomyces cerevisiae*	33	6	1437	99.6	30	3	1440	99.8

[1]) In this table reducing power is always expressed as mg maltose.

It should be added that a special control experiment showed that no maltose disappeared in a non-inoculated yeast water maltose medium incubated at 30° C. for 16 days.

The results reported in Table I leave no doubt that the non-maltose-fermenting yeasts *Brettanomyces anomalus, Mycocandida parakrusei, Torulopsis dattila* and *Torulopsis utilis* consume under aerobic conditions quite considerable amounts of maltose. For *Torulopsis dattila* this means a confirmation of HOEKE's earlier result. On the other hand it may be concluded that there are yeasts which also under the conditions of the growth experiments do not attack maltose at all. This holds quite strictly for *Saccharomyces fragilis, Torula cremoris* and *Torula monosa*, where the decrease in maltose does not surpass the limits of the experimental error. As for *Zygosaccharomyces Marxianus, Saccharomyces exiguus* and *Saccharomycodes Ludwigii* the figures obtained after the period of 35 days show a consumption which, although probably not due to experimental error, is still so low that for practical purposes it may be neglected.

c. Respiration.

The results obtained in the investigation of the suitability of maltose as a substrate for the respiration of the various yeasts species are collected in Fig. 2—11.

The curves reproduced in Fig. 2 to 5 leave no doubt, that *Brettanomyces anomalus, Mycocandida parakrusei, Torulopsis dattila* and *Torulopsis utilis* are, indeed, able to use maltose as a respiration substrate.

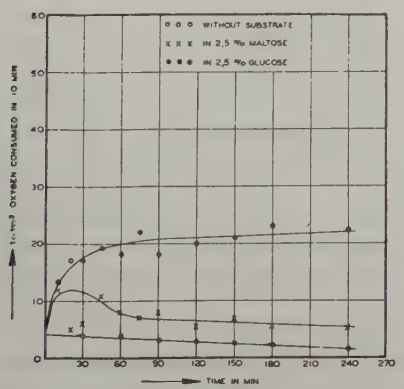

Fig. 2. *Brettanomyces anomalus* — Respiration with glucose, with maltose, and without substrate.

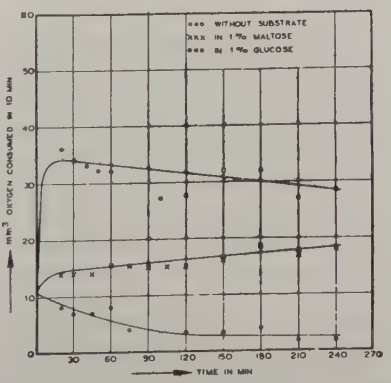

Fig. 3. *Mycocandida parakrusei* — Respiration with glucose, with maltose, and without substrate.

For the remaining six species (Fig. 6—11) it is clear that the initial increase in rate of oxygen consumption which follows the addition of maltose to the cell suspensions is due to the presence of a small amount

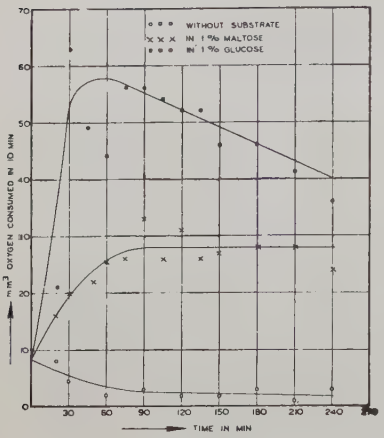

Fig. 4. *Torulopsis dattila* — Respiration with glucose, with maltose and without substrate.

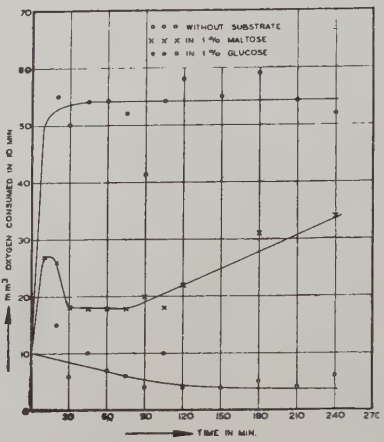

Fig. 5. *Torulopsis utilis* — Respiration with glucose, with maltose and without substrate.

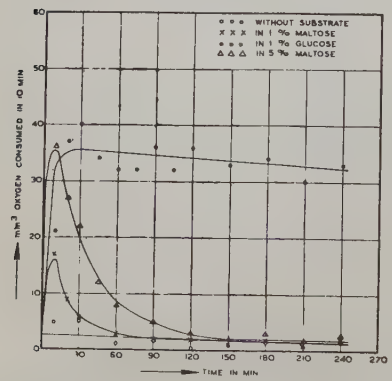

Fig. 6. *Zygosaccharomyces Marxianus* — Respiration with glucose, with maltose and without substrate.

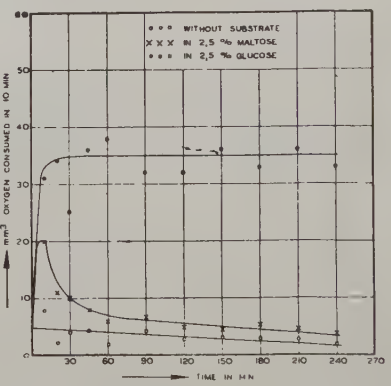

Fig. 7. *Saccharomyces exiguus* — Respiration with glucose, with maltose and without substrate.

of impurities in the sugar preparation. For after a relatively short time the respiration rate drops again to the value of the endogenous respiration. It will be superfluous to stress that these results are in highly satisfactory agreement with what might have been expected on

the basis of the results obtained in the assimilation experiments.

It is worth-while to draw attention here to the fact that the behaviour of *Zygosaccharomyces Marxianus* and *Saccharomyces exiguus* fully confirm the results reported by KLUYVER and HOOGERHEIDE

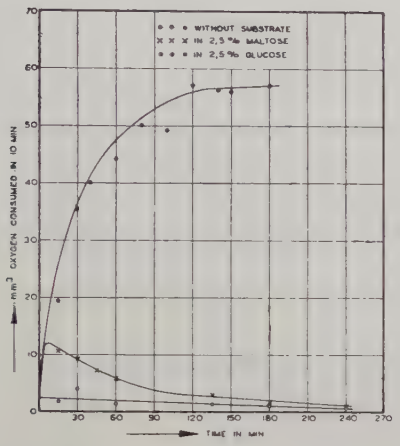

Fig. 8. *Saccharomyces Ludwigii* — Respiration with glucose, with maltose and without substrate.

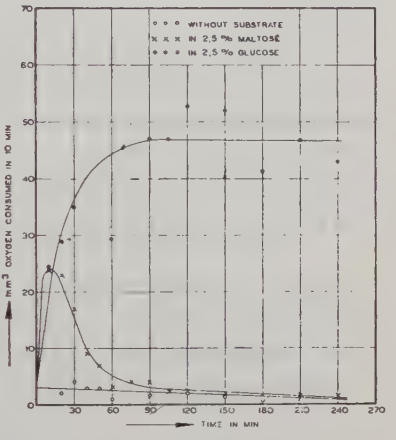

Fig. 9. *Torula cremoris* — Respiration with glucose, with maltose and without substrate.

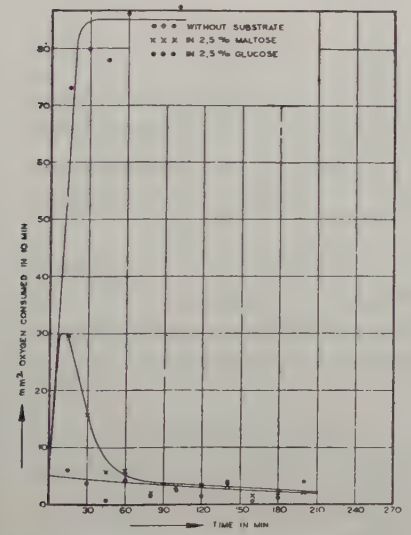

Fig. 10. *Saccharomyces fragilis* — Respiration with glucose, with maltose and without substrate.

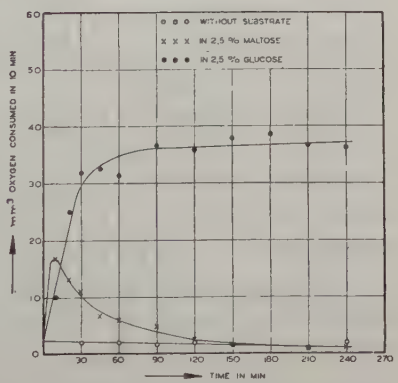

Fig. 11. *Torula monosa* — Respiration with glucose, with maltose and without substrate.

(17) regarding the non-suitability of maltose as a respiration substrate for these yeasts [1]).

By far the most important conclusion which must be drawn from these respiration experiments is, however, that for the first time convincing experimental proof has been obtained that, indeed, for certain yeast species which do not at all ferment maltose, this disaccharide can act as a substrate for the respiration.

We shall refrain here from a discussion of this remarkable fact, and of its bearing on our insight into the mechanism of respiration and fermentation. This discussion will be postponed to Chapter 6, and we shall first consider the corresponding results obtained in the case of the various yeast species which do not ferment lactose or saccharose.

4. RESULTS OBTAINED WITH THE NON-LACTOSE-FERMENTING YEAST SPECIES.

In the following an account will be given of the behaviour of the various yeast species enumerated in the list given in Chapter 1 under B.

a. Fermentation.

Here again it may be briefly stated that for all three species investigated all tests on their ability to ferment lactose, indeed, yielded completely negative results.

b. Assimilation.

The qualitative test for the assimilability of lactose-with the aid of the auxanographic method gave a clearly positive result with *Blastodendrion intermedium* which is a confirmation of the observation of Langeron and Guerra (l.c.). The two other species were completely negative in this respect.

The results obtained in the growth experiments with quantitative determination of the lactose are collected in Table II.

It should be added that a special control experiment showed that no lactose disappeared in a non-inoculated yeast water lactose medium incubated at 30° C. for 16 days.

From Table II it is evident that the non-lactose-fermenting yeast *Blastodendrion intermedium* consumes under aerobic conditions quite a considerable amount of lactose. It further appears, that neither the

[1]) The same cannot be said for the results published by Hoogerheide in his thesis (12) regarding *Torulopsis dattila*. Hoogerheide reports here that for this species too he did not observe maltose respiration.

Table II.

Lactose consumption in growth experiments with non-lactose-fermenting yeast species.

The initial reducing power of the yeast water present in the culture media was equivalent to 20 mg lactose, the initial reducing power of the lactose-containing media was equivalent to 1468 mg lactose; each culture flask, therefore, initially contained 1448 mg lactose.

Yeast species	After 16 days incubation at 30°C.				After 35 days incubation at 30°C.			
	Reducing power [1]) of the medium	Lactose remaining in medium	Lactose consumed in mg	Lactose consumed in % of initial amount	Reducing power [1]) of the medium	Lactose remaining in medium	Lactose consumed in mg	Lactose consumed in % of initial amount
Blastodendrion intermedium . .	—	—	—	—	356	336	1112	76.8
Saccharomyces carlsbergensis . .	1448	1428	20	1.4	1456	1436	12	0.8
Saccharomyces cerevisiae	1438	1418	30	2.1	1449	1429	19	1.3
Control with lactose-fermenting yeast: *Saccharomyces fragilis* .	20	0	1448	100.0	—	—	—	—

[1]) In this table reducing power is always expressed as mg lactose.

bottom yeast species, *Saccharomyces carlsbergensis*, nor the top yeast, *Saccharomyces cerevisiae*, is able to consume lactose to any appreciable extent, even although the experiments were extended over 35 days.

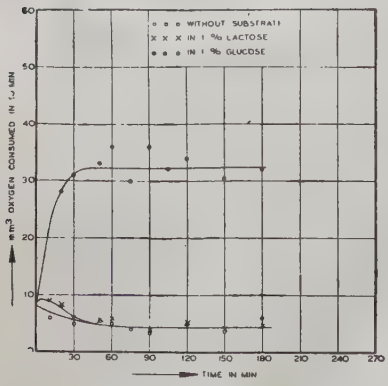

Fig. 12. *Saccharomyces carlsbergensis* — Respiration with glucose, with lactose, and without substrate.

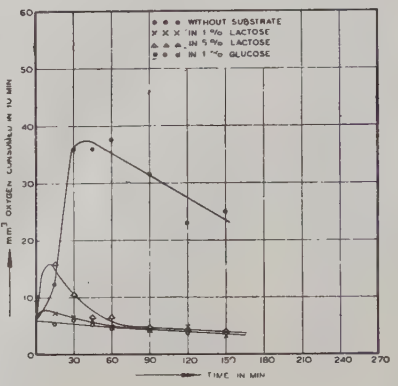

Fig. 13. *Saccharomyces cerevisiae* — Respiration with glucose, with lactose, and without substrate.

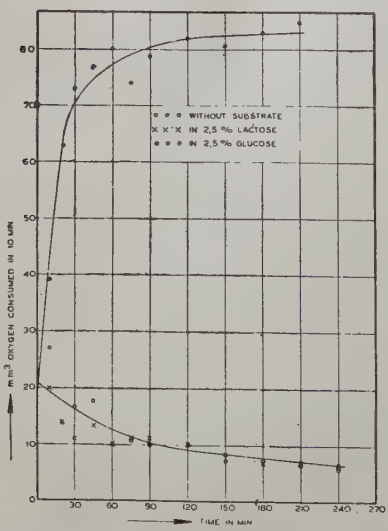

Fig. 14. *Blastodendrion intermedium* — Respiration with glucose, with lactose, and without substrate.

These results should be considered in connection with the observations made by HOFMANN and by PASTEUR referred to in Chapter 1.

A discussion of these results will, however, be postponed to Chapter 6.

c. Respiration.

The results obtained in the investigation of the suitability of lactose as a substrate for the respiration of the various yeast species are collected in Fig. 12—14.

As will be seen from the graphs 12 and 13, the results for *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* are in substantial agreement with what might have been expected on the basis of the results obtained in the assimilation experiments. In the first place it may be remarked that the lactose preparation used is only slightly contaminated with easily oxidizable

impurities: the respiration rate in the 1% lactose medium being during the whole period almost equal to that which is observed in the experiment without lactose addition. At the same time this offers proof that lactose is unsuitable as a respiration substrate for these two species.

On the contrary the result obtained with *Blastodendrion intermedium* is quite unexpected, in so far as the rate of respiration is not at all increased by the addition of lactose. With a view to the fact that this sugar is readily assimilated (Cf. Table II) this result is surprising. We shall return to this question in Chapter 7.

A further discussion of the results obtained with the non-lactose-fermenting yeast species will also be postponed to Chapter 6.

5. RESULTS OBTAINED WITH THE NON-SACCHAROSE-FERMENTING YEAST SPECIES.

In the following an account will be given of the behaviour of the various yeast species enumerated in the list given in Chapter 1 under C.

a. Fermentation.

The results obtained in the tests for the fermentation of saccharose by the various yeast strains differ markedly from the corresponding results reported in the two foregoing chapters.

The remarkable fact presented itself that we could only confirm for one species, *viz.*, the also non-maltose-fermenting *Torula monosa*, the statement that this species does not ferment saccharose. With the eight other strains for which the same behaviour has been claimed in literature we have observed a weak, yet unmistakable fermentative power towards this sugar.

It may be remarked by the way that all these species — with the exception of *Mycocandida parakrusei* and *Brettanomyces anomalus* — are characterized by the fact that they ferment maltose quite vigorously. This fact is suggestive in connection with WEIDENHAGEN's well-known theory on the close relationship between maltase and saccharase (34).

There is, however, a rather considerable difference between the various species with regard to the intensity with which they ferment saccharose, as may be derived from the fact that in some cases the fermentation could only be demonstrated with the aid of part of the four methods applied in the investigation.

We shall, therefore, give here a short survey of the ways in which each of the supposedly non-saccharose-fermenting species behaved in the various tests.

1. *Saccharomyces italicus.* Although CASTELLI (l.c.) states quite explicitly that this yeast does not ferment saccharose, we have been able to demonstrate fermentation with all four methods applied. It is true that in the beginning the results in the Einhorn tubes were negative — which sufficiently explains CASTELLI's results —, but this situation changed as soon as we made some tests with especially heavy inoculations. By also applying the shaking technique regularly, quite strong carbon dioxide evolution could be observed within 4—6 days. Tests in Struyk flasks were also always positive, provided heavy inoculations were applied.

In agreement herewith the yeast also gave positive results in the quantitative apparatus, in so far as in a solution of 2% glucose + 2% saccharose in yeast water always an excess of carbon dioxide was produced over the amount produced in a solution of 2% glucose in yeast water. It should be added, however, that in no case a quantitative fermentation of the saccharose could be attained. It was further found that the excess of gas produced in the saccharose-containing medium depended on the strength of the inoculation.

The manometric method also yielded a positive result which was quite marked, when a higher yeast concentration was used [1]), as may be seen from Fig. 15.

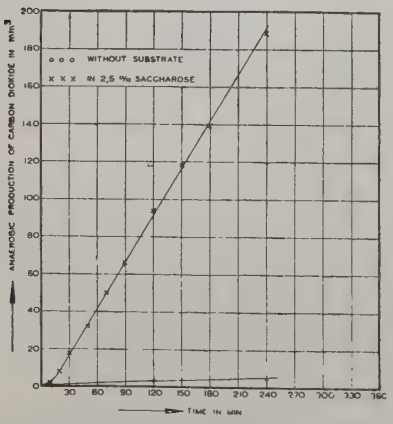

Fig. 15. *Saccharomyces italicus* — Fermentation of saccharose, and in the absence of substrate.

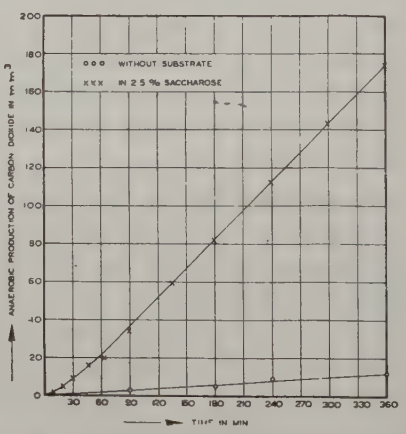

Fig. 16. *Brettanomyces anomalus* — Fermentation of saccharose, and in the absence of substrate.

[1]) In all the manometric experiments on saccharose fermentation reported in this chapter this high yeast concentration has been applied.

2. *Brettanomyces anomalus*. On the whole this species behaved like *Saccharomyces italicus*. In the Einhorn tubes the results were fully negative as long as the standard method was strictly adhered to. However, when applying abnormally heavy inoculations and the shaking technique a weak evolution of carbon dioxide could be observed. Also in the Struyk flasks a weak fermentation occurred, provided heavy inoculations had been made.

In the quantitative apparatus, using a solution of 2% glucose + 2% saccharose a complete fermentation of the saccharose could be obtained.

Also the manometric method gave a strongly positive fermentation, as may be seen from Fig. 16.

3. „*Torula* species CLAUSSEN". This strain also gave weakly positive results in all tests. In the quantitative apparatus only a small part of the saccharose added was fermented. The manometric method was, however, quite decisive in demonstrating fermentation, as may be judged from Fig. 17.

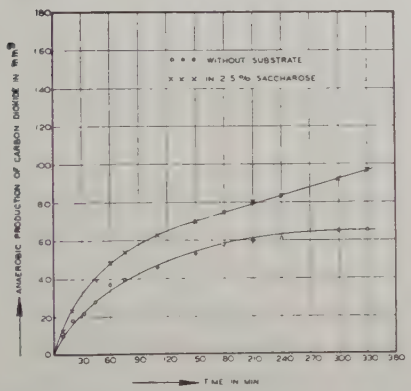

Fig. 17. *Torula* spec. CLAUSSEN — Fermentation of saccharose, and in the absence of substrate.

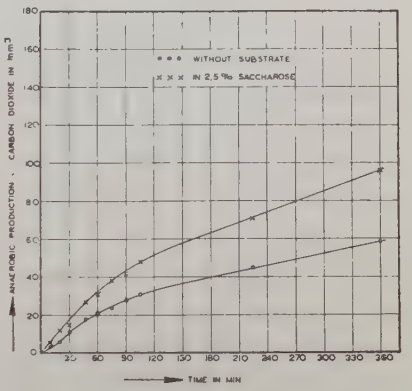

Fig. 18. *Mycotorula albicans* (strain 493) — Fermentation of saccharose, and in the absence of substrate.

4. *Mycotorula albicans* (strain 493). This strain gave, albeit very weakly, positive results both in Einhorn tubes and in Struyk flasks. However, in the quantitative apparatus no fermentative power towards saccharose could be ascertained. On the contrary the manometric method again revealed a weak but unmistakable fermentation, as may be seen from Fig. 18.

5. *Mycotorula albicans* (strain LEVY) showed a behaviour which is in substantial agreement with that of the preceding strain. However,

there were slight differences in so far as the fermentation both in the Einhorn tubes and in the Struyk flasks was more marked, whilst also in the quantitative apparatus a slight, although unmistakable, fermentation of the saccharose took place.

6. *Mycotorula albicans* (strain 417a SABOURAUD) behaved exactly like the foregoing strain.

7. *Mycocandida parakrusei*. Our first impression of the behaviour of this yeast was that it shared with *Torula monosa* the property of being incapable of fermenting saccharose under all conditions. However, it drew the attention that in the long run a small gas bubble could always be observed in the closed arm of the Einhorn tube. This induced us to prolonge the time of observation in the various tests, and this led to the result that after 15 days in the Struyk flasks always a quite marked fermentation occurred. The possibility of a retarded contamination could be excluded with certainty, and the phenomenon could be reproduced at will. On the contrary all attempts to ascertain fermentation of saccharose in the quantitative apparatus were unsuccessful. With the manometric method the results at first were negative, however, later when the experiments were repeated with a higher yeast concentration there was an unmistakable fermentation, as may be seen from Fig. 19.

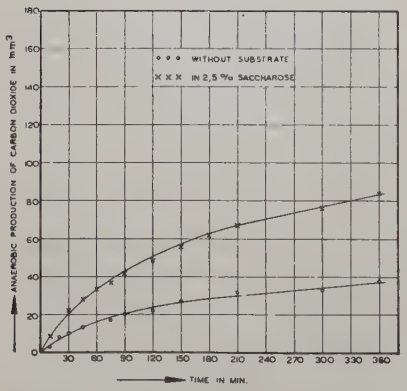

Fig. 19. *Mycocandida parakrusei* — Fermentation of saccharose, and in the absence of substrate.

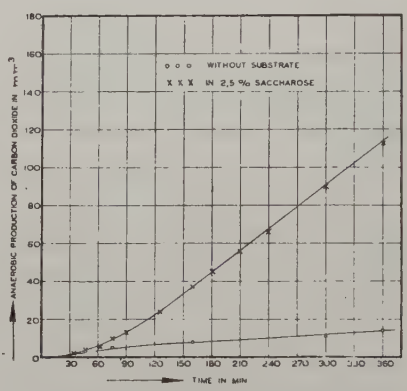

Fig. 20. *Schizosaccharomyces octosporus* — Fermentation of saccharose, and in the absence of substrate.

8. *Schizosaccharomyces octosporus*. In good agreement with BEIJERINCK's explicit statements the tests in the Einhorn tubes and in the Struyk flasks were completely negative, although these tests were

repeated several times. In contrast hereto the investigation in the quantitative apparatus showed an unmistakable fermentation, as appears from the figures for carbon dioxide production given below. In order to make sure that the fermentation of the saccharose was not due to a previous inversion during the sterilization of the medium, a parallel test was made with *Torula monosa*.

	Yeast water 2% glucose	Yeast water 2% glucose + 2% saccharose
Schizos. octosporus . .	4.7 cc	6.4 cc
„ „ . .	—	9.1 cc
Torula monosa . . .	4.8 cc	4.8 cc

Characteristic of the saccharose fermentation by *Schizos. octosporus* is the considerable disagreement between the duplicate tests, a phenomenon which was also encountered in a control experiment. Obviously the amount of carbon dioxide produced from the saccharose is largely dependent on the strength of the inoculation. It should be remarked that, whilst the fermentation of the glucose was already finished after 2 days, the fermentation of the saccharose proceeded quite slowly; the gas volumes given are those which were read after 6 days.

The manometric method also revealed a distinct fermentative power, as may be seen from Fig. 20.

b. Assimilation.

The qualitative test for the assimilability of saccharose with the aid of the auxanographic method gave for all species tested, with the exception of *Torula monosa*, positive results. It should, however, be added that the saccharose auxanograms of *Schizosaccharomyces octosporus* and *Saccharomyces italicus* were very weak. With the latter species more success was obtained by using very heavy inoculations, and on application of the „gutter" method described in Chapter 2.

The results obtained in the growth experiments with quantitative determination of the saccharose are collected in Table III.

It should be added that a special control experiment showed that no saccharose was inverted in a non-inoculated yeast water saccharose medium incubated at 30°C. for 35 days.

From Table III it is evident that all the strains investigated, with the exception again of *Torula monosa*, consume saccharose under

Table III.

Saccharose consumption in growth experiments with non-saccharose-fermenting yeast species.

The initial reducing power of the saccharose-containing culture media before inversion was equivalent to 25 mg invert sugar, the initial reducing power of this media after inversion was equivalent to 1560 mg invert sugar; each culture flask, therefore, initially contained 1458 mg saccharose.

Yeast species	After 16 days incubation at 30°C.					After 35 days incubation at 30°C.				
	Reducing power [1]) of the medium before inversion	Reducing power of the medium after inversion	Saccharose remaining in medium	Saccharose consumed in mg	Saccharose consumed in % of initial amount	Reducing power of the medium before inversion	Reducing power of the medium after inversion	Saccharose remaining in medium	Saccharose consumed in mg	Saccharose consumed in % of initial amount
Brettanomyces anomalus	20	1047	976	482	33.1	16	20	4	1454	99.7
Mycocandida parakrusei	46	883	795	663	45.5	43	846	763	695	47.6
Torula monosa	25	1543	1442	16	1.1	26	1547	1445	13	0.9
Saccharomyces italicus	18	93	71	1387	95.1	17	66	47	1411	96.8
Mycotorula albicans (strain 493) .	21	51	28	1430	98.1	16	26	9	1449	99.4
Mycotorula albicans (strain LEVY)	20	942	876	582	39.9	—	—	—	—	—
Mycotorula albicans (strain 417a SABOURAUD)	30	218	179	1279	87.7	—	—	—	—	—
Torula spec. CLAUSSEN.	—	—	—	—	—	17	46	28	1430	98.1
Schizosaccharomyces octosporus . .	—	—	—	—	—	21	339	302	1156	79.3
Control with saccharose-fermenting yeast: *Saccharomyces cerevisiae*	15	18	3	1455	99.8	—	—	—	—	—

[1]) In this table reducing power is always expressed as mg invert sugar.

aerobic conditions quite readily. The only remark to be made is that for *Schizosaccharomyces octosporus* it proved necessary to apply a heavy inoculation with a young and vigorous culture.

c. Respiration.

The results obtained in the investigation of the suitability of saccharose as a substrate for the respiration of the various yeast species are collected in Fig. 21—27.

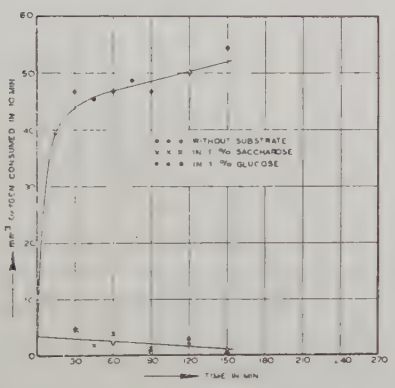

Fig. 21. *Torula monosa* — Respiration with glucose, with saccharose, and without substrate.

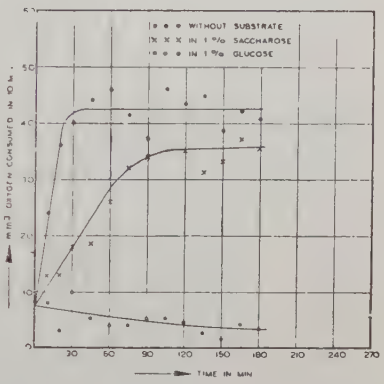

Fig. 22. *Mycotorula albicans* (strain 493) — Respiration with glucose, with saccharose, and without substrate.

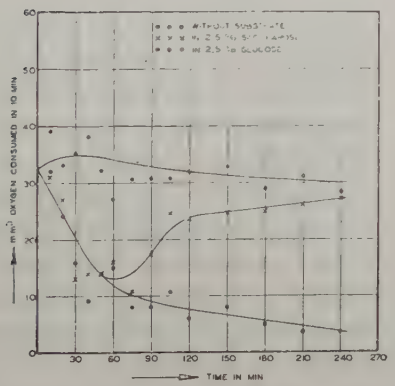

Fig. 23. *Torula* spec. Claussen — Respiration with glucose, with saccharose, and without substrate.

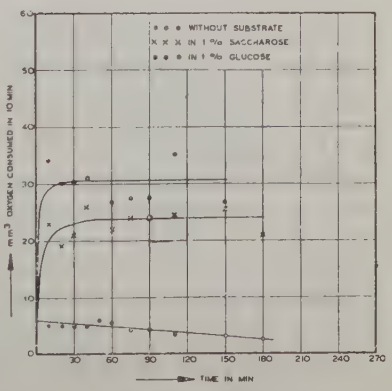

Fig. 24. *Mycocandida parakrusei* — Respiration with glucose, with saccharose, and without substrate.

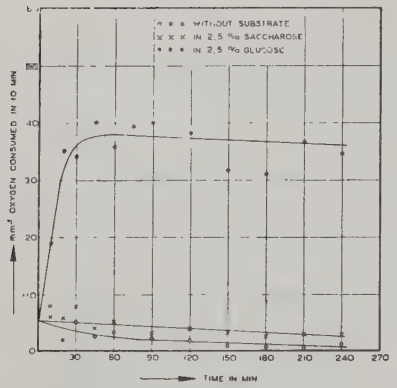

Fig. 25. *Schizosaccharomyces octosporus* — Respiration with glucose, with saccharose, and without substrate.

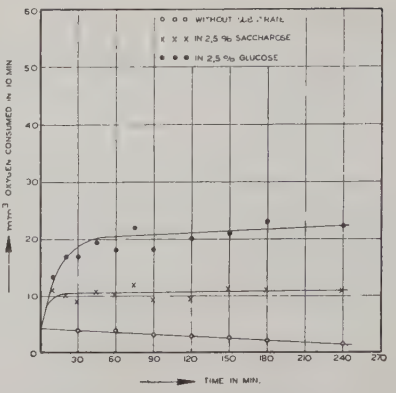

Fig. 26. *Brettanomyces anomalus* — Respiration with glucose, with saccharose, and without substrate.

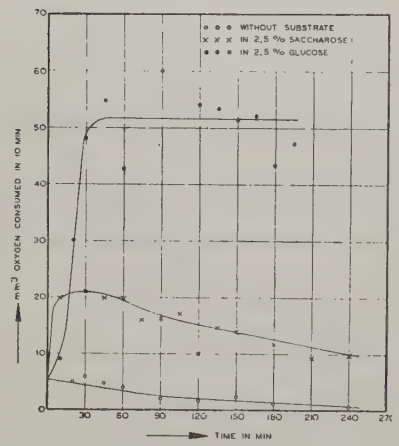

Fig. 27. *Saccharomyces italicus* — Respiration with glucose, with saccharose, and without substrate.

As will be seen from these graphs, the results are in substantial agreement with what might have been expected on the basis of the results obtained in the assimilation experiments. Evidently all strains, with the exception of *Torula monosa*, are able to use saccharose as a respiration substrate.

If we try to summarize briefly the results reported in this Chapter it should be stated that we have encountered one species, *viz.*, *Torula monosa*, which under all conditions does not attack saccharose. For all other species it has been found, however, that the statements in the literature according to which they do not ferment saccharose cannot be maintained. A weak fermentative power towards this sugar is undoubtedly present, and this fact seems significant for an interpretation of the marked ability of these yeasts to attack this sugar under aerobic conditions.

In the next Chapter, in which also the results obtained with the non-maltose-fermenting and the non-lactose-fermenting yeasts will be

considered, we shall enter into a more detailed discussion of this situation.

6. DO ASSIMILATION AND RESPIRATION OF UNFERMENTABLE DISACCHARIDES PROCEED WITHOUT PREVIOUS HYDROLYSIS?

In surveying the experiments dealt with in the 3 preceding chapters one result is quite conspicuous, namely, that a full confirmation has been obtained of the mostly very casual reports of earlier authors that certain yeasts have the ability to assimilate and to oxidize disaccharides which they do not ferment.

As has been remarked in Chapter 1 such a result cannot easily be understood. Until now it has quite generally been accepted that the inability of a yeast species — which ferments glucose — to ferment disaccharides like saccharose, maltose and lactose offered definite proof that these organisms do not contain the corresponding specific hydrolases, *in casu* saccharase, maltase and lactase. This conclusion stands to reason, since, if these hydrolases were present, and thus glucose would be split off, the disaccharides should also give rise to fermentation.

On the other hand it cannot be denied that the acceptance of the view that these disaccharides are assimilated and oxidized without previous hydrolysis meets with serious difficulties, as has already been set forth in Chapter 1. These difficulties are of such a grave nature that we do not hesitate to reject this idea.

Is there any possibility to escape from this impasse?

Now it seems to us that the results obtained in the investigation of the supposedly non-saccharose-fermenting yeasts lead the way to a better understanding. Here we have definite proof that the well-developed ability to assimilate and oxidize saccharose is accompanied by a weak, but in many cases quite distinct fermentative power towards this sugar. In these cases we have, therefore, more or less definite proof that the yeasts in question contain the corresponding hydrolase, the saccharase. For these species it is, therefore, not surprising that especially in experiments of long duration they are able to consume considerable amounts of saccharose.

Taking into account the difficulties connected with the demonstration of the saccharose-fermenting power — which explains the opposite reports of the earlier investigators — one can, however, not escape the conclusion that the activity of the saccharase is much greater under aerobic than under anaerobic conditions. In other words the facts

described point to the probability that under anaerobic conditions an inactivation of the saccharase, in this case only partial, occurs.

Once accepting this idea, it is tempting to apply this principle also to those cases in which assimilation and oxidation of a disaccharide takes place, although this sugar is not fermented at all. Here we must then conclude that under anaerobic conditions a complete inactivation of the hydrolase occurs.

It will not need comment that the views exposed above are quite contrary to current opinion. For instance we do not think that many investigators are willing to subscribe to the opinion that certain yeasts which do ferment glucose, but do not ferment maltose, nevertheless contain maltase which is completely inactivated under the conditions of fermentation, *i.e.*, under strictly anaerobic conditions.

We have, therefore, realised that it was necessary to support the hypothesis put forward in the foregoing considerations by more direct experimental evidence which is reported below.

The experiments made in order to arrive at a better understanding of the phenomena described in the preceding chapters have been chiefly made on *Torulopsis dattila*. The choice of this species seemed especially suitable, since in almost 20 years of biochemical sugar determination in this laboratory this yeast has amply proved its lack of ability to ferment maltose, whilst on the other hand both HOEKE's experiments and our own leave no doubt that it attacks maltose under aerobic conditions quite readily.

We have now set it our task to give proof that the last mentioned quality is due to the presence of a non-negligible amount of maltase in the yeast which, however, is inactivated under anaerobic conditions.

Taking into account that *Torulopsis dattila* is a strongly fermenting species which with glucose as a substrate shows a marked aerobic fermentation (12), it seemed possible that the maltase activity under aerobic conditions would be sufficiently large to yield also an aerobic fermentation with maltose as a substrate. If this would be the case direct proof would have been given that this supposedly non-maltose-fermenting yeast can actually ferment maltose, if only the inactivation of the maltase by the anaerobic conditions is avoided.

The experiment made to test this question has been reproduced in Fig. 28.

In this experiment the difference between the amount of carbon dioxide produced and the amount of oxygen consumed undoubtedly surpasses the limits of the experimental errors. We may, therefore,

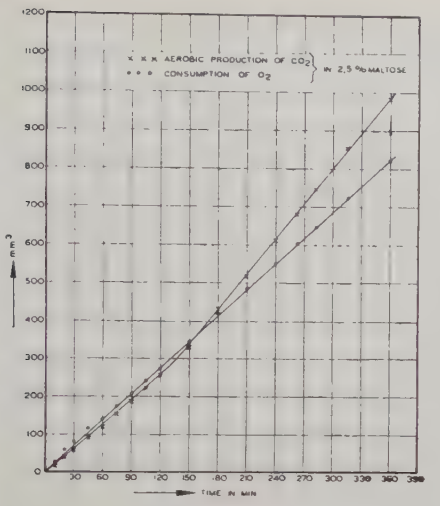

Fig. 28. *Torulopsis dattila* — Aerobic fermentation of maltose.

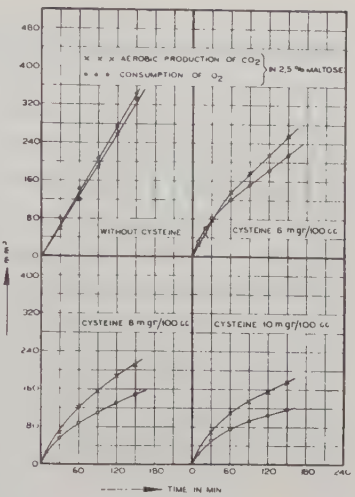

Fig. 29. *Torulopsis dattila* — Increased aerobic fermentation of maltose by addition of cysteine.

conclude to the occurrence of a distinct aerobic fermentation of maltose.

Encouraged by this result we have considered the possibility of increasing this aerobic fermentation of maltose by *Torulopsis dattila*. Hereto it was indicated to eliminate the PASTEUR–MEYERHOF effect by specific inhibition of the respiration.

We, therefore, decided to study the influence of increasing amounts of KCN on the aerobic fermentation of maltose. Here, however, we met with serious difficulties.

The low values of the respiration and the aerobic fermentation in a maltose medium made it necessary to continue the measurements over a period of at least three to four hours. Now it appeared that during the experiments the concentration of HCN in the medium — this time a phosphate buffer pH = 5.9 was used — decreased markedly in those vessels in which KOH was present for the carbon dioxide absorption. A lowering of the concentration of the KOH to 2.5% did not eliminate this trouble. Under these conditions it was impossible to measure the gas metabolism accurately. Nevertheless the expected effect could be clearly established in a qualitative way. It was found, namely, that at the lower concentrations of KCN, in the vessels without KOH the manometer indicated a marked increase in pressure, whilst in the experiment to which no KCN had been added a decrease in pressure — in accordance with the low value of aerobic fermentation — occurred. This

can only be due to the fact that the aerobic fermentation was increased by the KCN addition [1]).

Since, however, for the reason outlined above no measurements of the change in gas metabolism could be made, we have undertaken a series of experiments, in which cysteine instead of KCN has been used. As has been shown by QUASTEL and WHEATLY (24), by HOOGERHEIDE (l.c.) and others cysteine also inhibits the reaction of PASTEUR-MEYERHOF, and since this compound is not volatile, its use in experiments of longer duration seemed especially promising [2]). The results obtained in this series of experiments are collected in Fig. 29.

Fig. 29 shows that the addition of suitable amounts of cysteine results in an immediate occurrence of a quite pronounced aerobic fermentation. Here we have, therefore, conclusive proof that the supposedly non-maltose-fermenting yeast, *Torulopsis dattila*, is able to ferment maltose.

This result is especially striking, if one compares it with the fully negative result of the fermentation experiment under anaerobic conditions as reproduced in Fig. 1.

For us the results of the cysteine experiments is equivalent to a demonstration of the presence of maltase in this yeast, since we have always been unable to understand what other investigators mean when they defend the thesis of a so-called „direct fermentation" of maltose (Cf. for instance (20)). In our opinion this term should imply that in the fermentation of this disaccharide intermediate compounds with a number of C atoms between 7 and 11, and still containing the oxygen bridge of the maltose, will occur. Experimental evidence seems to be quite opposed to this supposition.

Leaving aside this problem, we have felt it desirable to give more direct proof for the presence of maltase in this non-maltose-fermenting yeast.

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[1]) The increase in pressure observed in the vessels with KCN is not due to a volatilisation of the HCN from the medium, as might be supposed. A simple calculation showed that the increase observed greatly surpassed the increase which would occur, if all the HCN added would have appeared in the gas phase.

[2]) In a recent paper KIRBY, DRILL and FREY (14) give results from which it is concluded that the effect of cysteine on aerobic fermentation is mainly due to H_2S arising from the action of yeast on this compound. Although in our case this possibility is quite immaterial, we have repeated some experiments with a lead nitrate solution in the well, in the way as described by the said authors. In the case of *T. dattila* no appreciable effect of this measure could be observed.

It seemed indicated to apply hereto the method designed by WILLSTÄTTER and BAMANN (36) for the estimation of maltase in maltose-fermenting yeasts.

Before giving here a survey of the results obtained a few general remarks should be made regarding the reliability of the usual methods for the demonstration of hydrolases in living cells. Usually these methods are very simple. Either the living cells as such, or dried preparations made from these are brought in a medium containing a phosphate buffer and the substrate to be tested, whilst at the same time a narcotic like toluene, chloroform or ethyl acetate is added. The narcotic should then completely inhibit the oxidoreductive removal of the products formed in the hydrolysis of the substrate, whilst the hydrolases themselves are insensitive to narcotic action. The products of the hydrolysis can then usually be detected without difficulty either by means of the polarimetric, or of a chemical method.

Now these methods are very useful, but there is one source of error which is too often neglected. Non-microbiologically trained investigators never consider the possibility that, notwithstanding the presence of a narcotic of the type referred to, such a medium can give rise to the development of contaminating micro-organisms which may seriously interfere with the results of the test. We have actually observed that this danger is quite real, in so far that in a maltose phosphate medium in the presence of toluene and ethyl acetate a quick development of lactic acid bacteria took place within 16 hours at 30°C., if ordinary bakers yeast of a high quality was added to the medium. In this case part of the glucose formed by the maltase of the yeast had been consumed by the bacterium.

In other cases in which the test organism does not contain maltase, it is quite conceivable that a contaminating organism may be responsible for the disappearance of maltose.

These short remarks may suffice to explain why in our opinion tests for the presence of hydrolases should always be accompanied by a rigid bacteriological examination of the medium at the end of the experiment. To us it is not doubtful that HOFMANN's demonstration of the presence of lactase in brewers bottom yeast — which is quite contrary to our experience as described in chapter 4 — must be ascribed to the fact that this author employed technical samples of this yeast in stead of a pure culture. His remark that this yeast initially only contained very few bacteria is of no importance, since in the long duration of his experiments an accumulation of one or more contaminating organisms

will certainly have occurred. Also in the work of many other investigators, neglect of this point of view will to all probability have often given rise to incorrect conclusions.

It will be superfluous to state here explicitly that in our own experiments for the demonstration of hydrolases full attention has been given to this question.

As for the demonstration of maltase in *Torulopsis dattila* the following can be reported.

The yeast was grown on malt agar plates. After incubation at 30°C. for two days the cells were scraped from the plates and washed. After centrifugation about 3 cc ethyl acetate and 10 drops of toluene were added to 12 grams of moist yeast. After 10 to 15 minutes standing at 30°C. the yeast was transported into 150 cc phosphate buffer pH = 7.2 to which 5% maltose was added. Both at the beginning of the experiment and at the end — after 23 hours — 30 cc of the medium was taken for analysis and mixed with 6 cc of a 2 n solution of sodium carbonate, in order to stop the reaction and eliminate mutarotation. Hereupon the yeast was removed by centrifugation, and in the clear solution both the optical rotation and the reduction of Fehling's solution were determined.

It was found that the optical rotation had decreased from 10.39° to 9.78°, whilst the Fehling reduction showed an almost corresponding increase. The polarimetric determinations indicated that 10.0% of the maltose initially present had been hydrolized into glucose. On the basis of the chemical determination the hydrolysis was found to be 11.9%, an agreement which may be deemed quite satisfactory taking into account the errors inherent in the determinations.

We may consider these results as a definite proof that the non-maltose-fermenting yeast *Torulopsis dattila* indeed contains a certain amount of maltase, the activity of which is then obviously fully lamed under anaerobic conditions.

The above mentioned observations regarding *Torulopsis dattila* make it extremely probable that the same situation will hold in the case of the abnormal behaviour of all yeasts which attack disaccharides under aerobic conditions, and yet either do not at all ferment these sugars, or do so only very weakly. This means that the anomaly in question is due to the fact that these species only contain such small quantities of hydrolases that the capacity of these primary catalytic agents limit the rate of respiration and of fermentation, whilst, more-

over, under anaerobic conditions a more or less complete inactivation of these catalysts takes place.

It would have led us too far to give complete experimental proof for the correctness of this extrapolation. We have restricted ourselves to a few test cases, about which we will briefly report in the following.

For the non-maltose-fermenting and — according to the data in literature — non-saccharose-fermenting species, *Mycocandida parakrusei*, we have first established that an aerobic fermentation indeed occurs with both maltose and saccharose, as is clearly shown in Fig. 30 and 31.

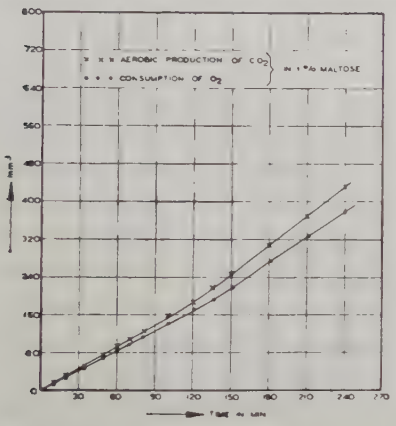

Fig. 30. *Mycocandida parakrusei* — Aerobic fermentation of maltose.

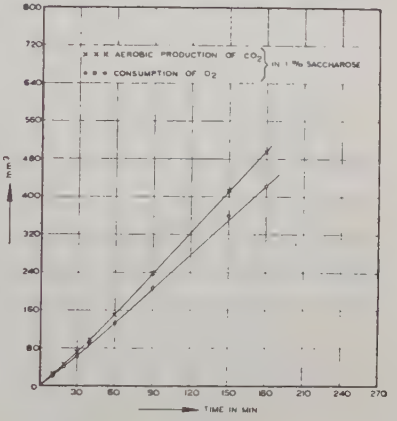

Fig. 31. *Mycocandida parakrusei* — Aerobic fermentation of saccharose.

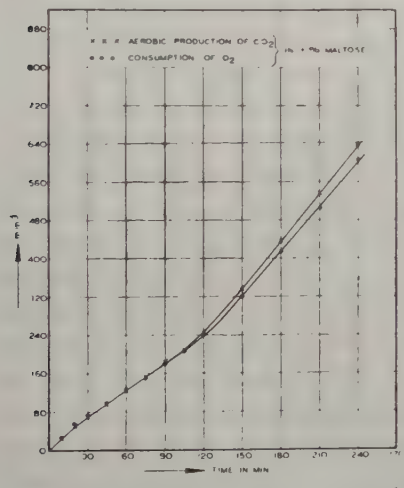

Fig. 32. *Torulopsis utilis* — Aerobic fermentation of maltose.

However, attempts to increase this aerobic fermentation by the addition of cysteine were unsuccessful. There was a marked production of H_2S from the cysteine, but remarkably the respiration was not inhibited.

The presence in this yeast of a small amount of saccharase could be demonstrated in a direct way with the aid of the method of WILLSTÄTTER, SCHNEIDER and BAMANN (35). A slight but unmistakable inversion of the saccharose was observed.

Although with some difficulty

the presence of maltase in this yeast could also be demonstrated.

As for *Saccharomyces italicus* we have been able to prove the presence of a slight amount of saccharase.

For *Torulopsis utilis* we have confined ourselves to a demonstration of a non-negligible amount of aerobic fermentation of maltose, as is shown in Fig. 32.

Although in this experiment the difference between the amounts of carbon dioxide produced and of oxygen consumed is only small, it undoubtedly surpasses the limits of the experimental errors.

Finally we have confirmed the earlier observation of HOFMANN regarding the presence of saccharase in *Schizosaccharomyces octosporus*.

7. ADAPTATION AS A FACTOR INFLUENCING THE BEHAVIOUR OF THE YEASTS IN THE VARIOUS TESTS.

In the preceding chapter we have given several arguments in favour of the view that, if a certain yeast species which does not ferment a disaccharide, nevertheless, is able to assimilate such a sugar, this is due to the circumstance that the yeast in question does contain a small amount of the corresponding hydrolase which, however, is inactivated under anaerobic conditions. A strong support for this idea is that for all yeast species which assimilate saccharose, but supposedly do not ferment this sugar, it could be shown that in reality they have a slight fermentative power towards saccharose. Here we must, therefore, conclude that there is only an incomplete inactivation of the saccharase by the anaerobic conditions.

On the basis of these assumptions on the whole a satisfactory explanation of the various phenomena observed could be given.

However, there are a few observations which seemed not to be in full agreement with this theory. In this respect we wish to draw attention to the following points.

In reviewing the various fermentation tests it is clear that a given yeast species does not in all cases behave in the same way. There are instances in which the Einhorn tubes and the Struyk flasks indicated fermentation, whilst in the quantitative apparatus no fermentation of the disaccharide occurred. In the case of the saccharose fermentation by *Schizosaccharomyces octosporus* the opposite behaviour was observed.

Now this divergence must certainly be partly ascribed to the different growth conditions prevailing in the various apparatuses. However, in certain cases one gets the impression that the cells of one and the same yeast show a different behaviour depending on their previous

history. In our trend of thought this should mean that the amount of hydrolases present in the cells of such a yeast may appreciably vary, or in other words that we are dealing here with a process of adaptation.

In the same way adaptation may well be responsible for some anomalies observed in the behaviour of certain yeast species under aerobic conditions.

We refer here to the fact that *Blastodendrion intermedium* did not show oxidation of lactose with the manometric method, although both the qualitative auxanographic method and the quantitative growth experiment gave clearly positive results.

Likewise *Schizosaccharomyces octosporus* showed only a very weak oxidation of saccharose with the WARBURG technique, although — using strong inoculations — the saccharose consumption in the growth experiments was very marked.

Finally, in the case of *Saccharomyces italicus* we met with considerable difficulties in our attempts to obtain a positive saccharose auxanogram. Nevertheless, the growth experiments gave proof that saccharose was nearly quantitatively consumed within a short time.

These observations also strongly suggested that adaptation was mainly responsible for these anomalies.

Now it is well known that there exists an extensive literature on the subject of enzymatic adaptation, for which we refer here to the recent review of KARSTRÖM (13). Especially for many hydrolases it has been shown that they belong to what KARSTRÖM has called „die nicht ausgeprägt adaptiven Enzyme", in so far as these enzymes are usually produced in slight quantities under very different nutritional conditions, but in larger amount, if the cells have grown in the presence of the specific substrate.

Accepting this to be the case also in our experiments, it is only logical to assume that the adaptation will occur to a quite different degree under the different conditions of the various tests.

We have set it our task to submit this adaptation hypothesis to an experimental test.

Since in all our experiments the yeast had been cultivated on malt agar, containing a high content of maltose, no success could be expected from adaptation experiments on maltase production. In this respect, it is significant, that with maltose as a substrate the various tests showed a quite satisfactory agreement.

Therefore, only adaptation experiments with saccharose and lactose seemed to offer prospects.

In the first place we have cultivated *Blastodendrion intermedium* on a medium consisting of yeast extract agar with 2% lactose. The growth of the yeast was very satisfactory. With the cells obtained from the lactose plates a respiration experiment in the Warburg vessel was made. The results are reproduced in Fig. 33.

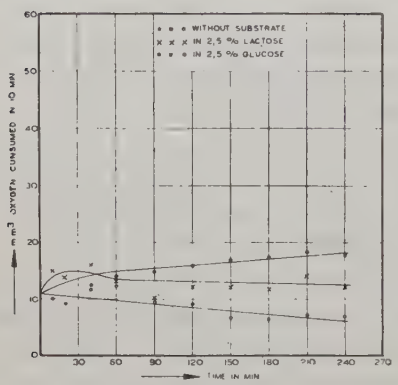

Fig. 33. *Blastodendrion intermedium* — Respiration with glucose, with lactose, and without substrate. All after adaptation to lactose.

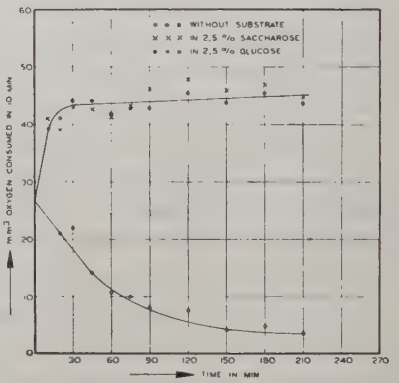

Fig. 34. *Schizosaccharomyces octosporus* — Respiration with glucose, with saccharose, and without substrate. All after adaptation to saccharose.

We see that with the „adapted" yeast there is a quite unmistakable respiration with lactose as a substrate, whilst in the case of the „unadapted" yeast no such a respiration could be established (Cf. Fig. 14).

In the same way we have tested the possibility to adapt *Schizosaccharomyces octosporus* to saccharose. Whilst we have seen that with the „unadapted" yeast cells from malt agar the respiration with saccharose was only very weak and remained far below the respiration with glucose (cf. Fig. 25), we find that the „adapted" cells show an equal rate of respiration with both sugars, as is clearly shown by Fig. 34.

8. FINAL DISCUSSION.

An analysis of the results reported in the preceding chapters leads to the conclusion that all yeasts which assimilate and oxidize disaccharides assumed to be unfermentable, nevertheless contain in greater or lesser quantity the corresponding hydrolases. However, under anaero-

bic conditions these hydrolases are inactivated either completely, or at least to such an extent that the fermentability of the disaccharide is not detected by the relatively insensitive routine methods for the determination of this property.

It will be clear that this conception removes the difficulties inherent in the at first sight quite abnormal behaviour of the yeast species in question, a behaviour which seemed to suggest that disaccharides were assimilated and oxidized without previous hydrolysis.

By establishing that certain yeast species do not at all ferment a disaccharide, although the corresponding hydrolase has been definitely shown to be present in the cells, the conclusion seems warranted that this hydrolase is in some way completely inactivated by the anaerobiosis. However, the question of the way in which this inactivation takes place, remains to be answered.

In principle two possibilities ask for consideration.

Firstly it does not seem excluded that under anaerobic conditions the substrate — *in casu* the disaccharide — is for some reason or other unable to get access to the hydrolase, thus dooming the latter to inactivity. DIXON (7) has forwarded a similar explanation for the so-called PASTEUR-effect by assuming that the action of oxygen consists in a decreased accessibility of the glycolytic and respiratory enzymes to glucose, or in other words that oxygen would decrease the permeability of the cell for this substrate.

In our case an explanation of the inactivation of the hydrolases on the basis of such a conception would involve the acceptance of exactly the opposite effect, *viz.*, a decrease in permeability of the yeast cell for special disaccharides in consequence of the removal of oxygen.

However, many reports in literature are in favour of DIXON's assumption that exclusion of oxygen tends to increase the permeability of the cell. In his doctorate thesis BULT (2) cites several instances where such an increase of permeability was encountered in the case of animal cells. In the meantime the recent investigations of ARISZ and his collaborators — we refer here only to a recent survey of this work (1) — give clear proof that generalization in this respect is not at all allowed. In several cases the said investigators could observe that the uptake of various substances by plant cells was strictly bound to the presence of oxygen. Yet one should not loose sight of the fact that these results were obtained with cells deprived of any anaerobic dissimilatory power. Therefore, the case may be quite different for the facultative anaerobic yeast cells.

On considering our special case attention should be drawn to the following. Some time ago RUNNSTRÖM (28) and collaborators have claimed to have given experimental proof for a strong increase in permeability of yeast cells under anaerobic conditions as compared with aerobic conditions. However, in a quite recent publication of RUNNSTRÖM, BOREI and SPERBER (29) this statement has been withdrawn, and a different interpretation of the observed facts has been announced.

We must, therefore, conclude that the question of the influence of oxygen on the permeability of yeast cells has not yet definitely been settled.

Nevertheless, it is difficult to conceive that anaerobiosis should decrease the permeability of the yeast cells for the disaccharides under consideration. For these cells show a normal PASTEUR-effect with glucose or well-fermentable disaccharides as substrates, proving that by withdrawal of oxygen the permeability for these sugars either remains unchanged, or even — according to DIXON's conception — is considerably increased. Now, it seems extremely improbable that the cells behave quite differently towards supposedly unfermentable disaccharides as towards the other sugars mentioned.

On rejecting the permeability hypothesis we have to accept the second possibility, *viz.*, that the inactivation is due to a reversible change of the catalyst itself. Taking into account that withdrawal of oxygen inevitably leads to an increase in the state of reduction in the interior of the cell, it is only logical to assume that the said change is connected with a reduction process.

Until now no evidence for the idea that carbohydrases are inactivated by reducing agents is available. Yet it should be remembered here that for another group of hydrolases, namely for various proteinases, it has clearly been shown that the state of reduction greatly affects their activity. We only refer to the well-known case of papaine, and to that of the various kathepsines.

It will be remarked, however, that we are dealing here with quite the opposite case, in so far as reduction increases the activity of these enzymes which even can be completely — but reversibly — inactivated by oxidation. Yet this behaviour of many proteinases need not be incompatible with our idea that reduction leads to inactivation of carbohydrases present in the living cell. On the contrary, it seems quite possible that the increased state of proteolysis which is characteristic for the cell under anaerobic conditions is the very cause of the intra-

cellular inactivation of the carbohydrases which too are proteins, or at least contain proteins as main components.

It is obvious that only further investigation can settle this question.

We wish to thank here Mr. H. J. PHAFF for his enthousiastic and able co-operation in the performance of the earlier part of the experimental work reported in this paper.

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(From the Laboratories of Hygiene of the University; of the Department for Tropical Hygiene of the Colonial Institute, and of the Health Department of the Municipal Medical and Health Service, all at Amsterdam).

RECORD OF A CASE OF PSITTACOSIS

by

H. VERVOORT AND A. CHARLOTTE RUYS

(Received March 13, 1940).

In a previous publication (3) we stated that we did not succeed in isolating the virus of psittacosis, neither from a collection of suspected birds, nor from the sputum obtained during a light illness of one of the assistants, who worked in the same room where the birds were kept. When this paper was already in the press, however, the virus could be demonstrated in a passage obtained from the original mice inoculated with a suspension of the sputum. Smears made from the peritoneal fluid, the spleen and the pericardial fluid were positive. As conditions allowed a fairly complete examination of this case, we deemed it of possible interest to record our findings.

Birds. A collection of ten budgerigars was bought at the market on February 27th, 1939, and kept to be used for experimental purposes in the yellow fever section of the laboratory. On March 2nd a bird fell ill and was found dead on the following morning. On March 6th a second bird fell ill, and was killed. At the post mortem examination both birds were found to be very skinny; no suggestive findings; spleens apparently not enlarged (3—4 mm); no virus found in smears. No microbes were ascertained at the examination of a culture of spleen-tissue on blood-agar. Mice inoculated with a suspension of the spleens remained in good health. They were killed after 10 to 19 days. No suspect findings. Several transmissions were made into other mice; the results remained negative. The remaining eight birds were kept under control in a screened part of the room. They looked healthy, the necessary precautions were taken, no masks were used. As there was no direct suspicion of psittacosis, the birds were used for experi-

ments with yellow fever virus in two batches of four each: respectively on March 20th and May 1st, 1939.

The birds of the first batch were caught on ten subsequent days, and their blood tested during the period of March 21st to April 14th. One bird was droopy from April 10th to the 14th, but in good condition again afterwards. On May 2nd another bird showed the same symptoms, and on May 4th, the experiment being completed, the whole batch was killed. At the post mortem examination no suspect findings were ascertained, the birds were well fed, the spleens not enlarged, and there was no psittacosis virus in the smears. Six mice were inoculated with suspensions of the spleens and livers. Passages were made, but no virus was revealed.

The second batch of birds remained healthy and was used for experiments on May 1st; after which date the birds were not handled any more. They were killed on May 23rd; post mortem findings negative; no virus in smears; inoculation into eight mice; no illness. Transmissions proved negative.

Personal. Two members of the staff, an adjunct assistant (J. H.) and a helper (J.J.J.), worked in the same room where the birds were kept during a couple of hours daily, at a table which was standing against the screened part of the room. On May 9th a blood sample was taken from both workers for the complement-fixation test, simply by way of control, and at the same time also from Mrs. V., who daily worked with infected mice. The adjunct assistant's serum was positive, also the one obtained on May 19th ($^1/_4$ ++++, $^1/_8$ +++), (W. R. negative); while the other two serum samples were negative.

On May 19th the adjunct assistant (J.H.) mentioned that he had been feeling unwell since a fortnight, and complained of a sore throat, which proved to be slightly red. Since two days he had had a headache, with pain in the loins and the back; a feeling like influenza. His temperature was not taken. He continued his daily work. His sputum was collected for inoculation into mice, and a desinfecting gargarism was prescribed. A smear from the sputum was determined as suspect. Two mice were inoculated with a suspension of the sputum in peptone-broth, after it had been centrifuged at moderate speed during fifteen minutes. The following day (May 20th), two other mice were inoculated with the same suspension that had been kept in the refrigerator overnight, and had been centrifuged again at 1500 r.p.m. during fifteen minutes. Passages were made from the first mice. In the case of this experiment pneumococcus infection in a high degree proved to be an

obstacle. On June 18th the virus of psittacosis was revealed in smears made from the peritoneal and the pericardeal fluid, and from the spleen of a mouse inoculated with a mixed suspension of spleens from a mouse killed in the third passage and from one killed in the first passage. The transmissions are recorded in Table I.

On June 19th J.H., who had in the meantime recovered, again complained of a sore throat. No suspect findings were ascertained, however. But in order to make quite sure, a second sample of his sputum was collected and a suspension inoculated into two mice on the same and following days, with negative results. On June 30th a third sputum test was made, without revealing the virus.

The second member of the staff, (J.J.J.), felt ill on June 10th, *i.e.*, sixteen days after the last batch of birds had been killed. He had to stay at home for a week. No virus was revealed in a sample taken of his blood, nor in his sputum inoculated into mice. The results of the complement-fixation test carried out on June 26th and July 27th, 1939, were negative.

On February 2nd, 1940 the complement-fixation test made with blood taken from both workers was negative (Table II).

Comments. Though the results of the tests for the detection of psittacosis virus in the birds were negative, it is very likely that the latter were carriers of this virus. It is possible that the virus was too weak to develop in mice, or that it required special conditions to do so. Also transmissions from spleens of mice inoculated with three different strains, isolated from other birds and with a strain isolated from a fatal human case, for some reason or other proved unsuccessful on several occasions and did not reveal the virus. The human strain only became virulent in transmissions after nearly a year. At the 32nd passage in the white mouse, death occurred earlier and was stabilised at from 2 to 4 days. The bird-strains were lost. As to the lower virulence, BURNET (1) made the same observation in respect of recently caught Australian birds, and MEYER and EDDIE (2) observed the same in freshly imported Australian parrots. They found the complement-fixation test with serum taken from the birds of great value for detecting the existence of psittacosis among the birds.

On the other hand, the strain isolated from the sputum of J.H. proves to be of a high virulence. It kills a mouse in two to five days. When examined with the naked eye, the post mortems often show „typical" pathological changes in a higher degree than in the case of our first isolated „RUYS" strain.

Result of the examination of the spu

	direct suspension of the sputum	
No. of mouse:	1	2
inoculated:	May 19.	May 19.
symptoms:	May 20 deadly ill. killed (2nd day)	May 23 dead (5th day).
findings:	slimy fluid; distended bowels.	slimy fluid; clear bowels distended; spleen normal; nals red; infiltration o lungs
smear:	pneumococcus.	few pneumococcus. s u s p inoculation of a suspensi spleen after centrifugatio No. 5 and 6.
		↓
No. of mouse:	5	6
inoculated:	May 23.	May 23.
symptoms:	May 29 slightly ill. killed (7th day)	June 1 not ill. killed (10th
findings:	duodenum clear, not distended; spleen soft, slightly enlarged; adrenals red.	large spleen.
smear:	few pneumococcus, toxoplasm. s u s p e c t. inoculation of a suspension of spleen into No. 7 and 8.	toxoplasm. not suspect.
	↓	
No. of mouse:	7	8
inoculated:	May 29.	May 29.
symptoms:	May 30 deadly ill. killed (2nd day)	June 9 not ill. killed (12th
findings:	slimy fluid.	spleen and liver enlarged; h rhagic spots in lungs.
smear:	pneumococcus.	toxoplasm. s u s p e c t. inoculation into No. 9 a together with Nr. 4.
		↓ ←
No. of mouse:	9	10
inoculated:	June 9.	June 9.
symptoms:	June 11 deadly ill. killed (3d day).	June 18 slightly ill, dis belly. killed (10th day).
findings:	slimy fluid; distended bowels; spleen small; liver bluish; adrenals red.	a trace of slimy fluid; dis bowels; spleen and liver ed; adrenals red.
smear:	pneumococcus.	v i r u s o f p s i t t a c o present in peritoneal flu spleen.
		↓
		transmissions

J.H. by mouse inoculation

same suspension, kept in refrigerator and centrifuged

3	4
20.	May 20.
29 not ill. killed (10th day).	June 9 not ill. killed (21th day).
tive.	a trace of fluid in periton; large spleen.
pect.	toxoplasm. suspect.
	inoculation of a suspension of spleen, together with No. 8 into No. 9 and 10.

Table II.

Epidemiological data.

Birds

1939
Febr. 27: 10 budgerigars bought at the market.
March 3: one died
March 6: one ill and killed. } post mortem findings negative. No virus of psittacosis revealed in transmissions into mice.
May 4: 4 healthy, killed
May 23: 4 healthy, killed

Persons	exposure to infection			first date of illness	course of illness	laboratory findings		
	earliest date	degree	nature of work			serum complement-fixation test	sputum inoculated into mice	blood inoculated into mice
J.H.	1939 Febr. 27	direct	handled birds for experiments, worked in same room	between 5 and 10 May	light degree, recovered	1939 May 9 + May 19 + June 26 + 1940 Febr. 2 —	May 19 pos.	—
J.J.J.	Febr. 27	direct	idem, fed birds and cleaned cages	June 10	light degree, recovered	1939 May 9 — June 26 — July 17 — 1940 Febr. 2 —	June 19 neg.	June 14 neg.
H.V.	March 3	remote	daily inspection of birds; handled birds for post mortem and virus research	well		neg.		

J. H.'s illness was of a very mild nature, but the presence of the virus in his sputum, together with the positive complement-fixation test, which now again proved to be negative, support the diagnose. J.H. was only in contact with infected mice a year ago (May 1938). As it is impossible to fix the date of the infection between Febr. 27th and May 5th 1939, no suggestion can be made regarding the time of appearance of the positive complement-fixation test.

It was by mere chance that we succeeded in isolating the virus and getting rid of the pneumococcus infection. As one of us inoculated, fed, cleaned and handled the mice himself, no mistake in connection with glass-containers or labels can have occurred. Moreover, the coincidence of the pneumococcus infection in every group of inoculated mice proves the origin of the infection.

Discussion. This case of psittacosis infection should not be considered as a mere laboratory incident. What took place in the laboratory is a demonstration of what may happen in a family. A collection of birds is bought. Unfortunately, one or two of them fall ill, or die, a few days after the transport, but the remaining birds are healthy and the pride of their owners. On a certain day a member of the family, — most likely the one who takes care of the birds —, falls ill. It may be a slight cold, an affection of the throat, an attack of influenza, or even a severe pneumonia-like disease, from which it takes a long time to recover: it may also be fatal. May be only one member of the family is affected, may be more. And nobody has a notion of the fact that the cause of the disease entered the house together with the birds. This is just an example of what may happen, and a warning that there is always a risk in buying birds from an unknown source, *e.g.*, at the market, especially when new specimens are added to an old stock of birds.

The above case again proves that psittacosis infection must be endemic among home-bred birds, sold in Amsterdam.

It also proves the weak nature of the virus and the difficulty of isolating it by inoculation into mice, and at the same time it confirms the value of the complement-fixation test for the recognition of human cases.

It proves that a close co-operation between the physician and the laboratory, especially during the first days of the illness, is the only way to reveal the origin of the disease in time to prevent its spreading.

It equally proves that in all fever cases, even light ones, when the patient has been in contact with birds that are not entirely

beyond suspicion, the possibility of a psittacosis infection should be considered.

Summary.

The above is a record of a very mild case of psittacosis infection, recognised as such only by means of epidemiological considerations, simplified by developments as these occurred before our eyes. The results of laboratory tests on material obtained from the birds and from a human case, are recorded. The possibilities of infection by apparently healthy birds are discussed.

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(Laboratorio di Batteriologia Agraria della Facoltà Agraria della R. Università, Milano).

LE LAIT COMME MILIEU DE CULTURE EN BACTERIOLOGIE

par

C. GORINI

(Reçu le 27 mars 1940).

Pour un bactériologiste du lait la mémoire de LEEUWENHOEK est doublement vénérable: par la découverte du microscope et par la découverte des globules gras du lait (5). C'est pourquoi, désirant répondre à la flatteuse invitation de collaborer à la suggestive revue „Antonie van Leeuwenhoek", je me permets d'esquisser un éloge du lait comme milieu de culture microbienne, à partir de mes investigations quasi cinquantenaires sur la façon de se comporter des bactéries dans le lait, d'où sont sortis plusieurs faits nouveaux qui intéressent soit la physiologie et la biochimie des microbes, soit la connaissance intime du lait même. Je diviserai mon esquisse en différents chapitres.

1. LE LAIT EST UN MILIEU GÉNÉRAL DE CULTURE MICROBIENNE.

Le lait se prête à la culture de toutes les bactéries, y compris les pathogènes, non seulement parce qu'il constitue un milieu nutritif complet, mais aussi parce que, comme le sang, il contient des protéines natives et complexes dont les germes parasites sont si exigeants. Ce sont là des substances qu'on ne trouve certainement pas dans les milieux de culture ordinaires à base de peptones et de gélatines. En outre, le lait est riche en éléments minéraux biogéniques ainsi qu'en vitamines et en d'autres principes stimulateurs de croissance et d'activité. Il y a des bactéries pathogènes, tel que le *B. pyogenes bovis* (syn. du *B. minimum mammae* Gorini, 1907) qui, pendant quelque temps après leur isolement de l'organisme, se développent difficilement dans les milieux ordinaires, à moins qu'on ne leur adjoigne un peu de sang ou bien un peu de lait. De plus, lorsqu'on offre aux germes un substratum optimum

comme le lait, on peut avantageusement y ajouter les différentes matières sur lesquelles on désire étudier l'action de ces germes; tandis qu'au contraire, les recherches ne sont pas concluantes quand les additions sont faites à des milieux de culture moins favorables, tels que la gélatine, la gélose, le bouillon, etc.

Même les bactéries qui ne produisent aucune altération apparente dans le lait, peuvent y vivre et s'y conserver longtemps; je cite l'exemple du *B. radicicola* dont j'ai démontré la capacité de vivre dans le lait pendant six à dix mois (Dernièrement le lait a été déclaré le substrate de choix pour les Leishmanies, Bianchi (1)).

2. ENZYMOLOGIE BACTÉRIENNE.

Les lactocultures m'ont servi à l'étude de l'enzymologie bactérienne en décélant la chymase du *B. prodigiosum* et des bactéries acidoprésurigènes.

L'étude de la chymase, également produite sur les milieux ordinaires sans caséine, m'a permis de démontrer dès 1892:

a. l'existence d'enzymes thermostabiles jusqu'à 100°C. *b.* l'existence d'enzymes que j'appelle habituelles (enzymes constitutives de Karström) qui, contrairement aux enzymes adaptatives, sont produites par le microbe sur tous les substrates, même en absence de la substance spécifique. *c.* des différenciations entre la chymase microbienne et la chymase animale (action sur le lait cuit, thermostabilité, etc.).

L'étude des bactéries acidoprésurigènes m'a permis de démontrer dès 1892—1894, que, à côté des ferments simplement saccharolytiques ou simplement protéolytiques, il existe des ferments doubles ou acidoprotéolytiques qui attaquent simultanément les sucres et les albuminoïdes, ceci contrairement à l'opinion alors dominante selon laquelle la saccharolyse et la protéolyse seraient des procès antithétiques. Par là j'ai donné aussi le premier exemple de bactéries capables de peptoniser en milieu acide, tandis que jusqu'alors on croyait que les bactéries ne peptonisaient qu'en réaction alcaline ou tout au moins neutre.

Toutes ces conclusions sont, vraisemblablement à cause de leur précocité, restées inaperçues pendant plusieurs années, mais dans la suite l'étude en a été reprise et elles ont été confirmées et étendues par moi-même et par d'autres auteurs et aujourd'hui elles ont acquis une importance considérable aussi pour l'enzymologie générale.

Quant à la chymase on a vérifié, notamment par ma méthode de culture sur gélose-lait (1932—1934), que sa production est très répandue

chez toutes sortes de microbes (schizomycètes, blastomycètes et hyphomycètes) même parmi ceux qui sont considérés inactifs sur le lait (*B. typhi, Str. equi, Str. equines, Sacchar. cerevisiae, Actinomyces* 47 Lieske, et *Actinomyces asteroides* de la collection de Baarn, etc.).

Quant aux Acidoprotéolytes, on les a rencontré dans plusieurs milieux intéressant l'agriculture, l'industrie et la médecine, particulièrement là où s'accomplissent des protéolyses sans putréfaction (ensilage, fromagerie, tannerie, certains procès pathologiques, etc.). Par rapport à la fromagerie, les premiers à reconnaître la présence des Acidoprotéolytes ont été Boekhout et Ott de Vries (à savoir dans le fromage d'Edam (2)). Dernièrement, (1932) par la méthode moderne on a constaté que les Acidoprotéolytes produisent dans le lait des lyo- et des desmoenzymes, des protéinases et des peptidases, par quoi ils se distinguent des saccharolytes simples qui, ne formant pas des protéinases à côté des peptidases, sont incapables d'attaquer les protéines natives; ils n'attaquent que les protéines hydrolysées. D'autre part on a isolé chez les Acidoprotéolytes des acidoprotéases spéciales (Gorbach (3)) qui les différencient des alcalinoprotéolytes; en effet, cette distinction n'est pas possible dans les substrates ordinaires. Le *B. prodigiosum*, par exemple, qui dans le lait est un acidoprotéolyte, se comporte dans la gélatine comme un alcalinoprotéolyte à l'instar du *B. pyocyaneum*, tandis que ce dernier demeure alcalinoprotéolyte même dans le lait. Cela vient renforcer mon ancienne proposition (1894) de faire un groupement enzymatique des bactéries en trois classes, fondées sur leur action métabolique principale dans le lait, c'est-à-dire: 1. saccharolytes, 2. protéolytes, 3. acidoprotéolytes. Un tel groupement a de l'importance non seulement pour la science pure, mais aussi pour la science appliquée, soit la médecine ou la zymotechnique (V. mon Rapport au 18me Congrès de la Société allemande de microbiologie, Vienne 1939).

En outre, c'est par moyen des lactocultures qu'on a commencé à démontrer la différence d'action des germes sur les différents protéines, ainsi qu'il a été signalé par moi (1902) à propos du non-parallélisme entre la caséolyse et la collolyse, en ce sens, que les bactéries peptonisant le lait ne liquéfient pas toute la gélatine et vice versa. Aussi cette divergence a-t-elle été expliquée par voie enzymatique, puisqu'on a isolé des protéases bactériennes qui solubilisent seulement la gélatine ou seulement la caséine (Gorbach (3) et Maschmann (6)).

Je mentionnerai enfin la condition spéciale du lait favorisant la recherche d'autres enzymes microbiennes (lipases, oxydases, catalase, etc.).

3. INFLUENCE DES CONDITIONS DE L'AMBIANCE SUR LES FONCTIONS BACTÉRIENNES.

Les lactocultures se prêtent à étudier les principales exigences vitales des bactéries vis-à-vis de l'ambiance. Je me bornerai à considérer l'influence de la température, de l'air et de la nutrition.

a. Température. Les lactocultures des acidoprotéolytes démontrent qu'en général les températures élevées favorisent la saccharolyse alors que les températures basses favorisent la protéolyse (1897), par quoi certains acidoprotéolytes ne révèlent leur propriété protéolytique qu'à des températures basses, tandis qu'à des températures élevées ils peuvent passer pour de simples saccharolytes à cause d'une acidification excessive (acidité d'arrêt).

À part cela le lait peut être modifié par voie bactérienne à toutes les températures depuis —5°C. jusqu'à 70°C., de sorte que les lactocultures sont les plus aptes pour étudier les différentes exigences thermiques des bactéries et pour les distinguer, d'après Beijerinck, en psychrophiles, microthermes, mésothermes, mégathermes et thermophiles. Donc, le lait est un milieu d'élite pour les bactéries thermophiles; à partir de mon *B. lactis thermophilus* (1894) la plupart des nombreux bacilles nécessairement ou facultativement thermophiles ont été isolés du lait. Le lait est aussi un milieu d'élite pour démontrer que les bactéries peuvent être thermorésistantes même sans spores, grâce à la formation de manteaux protecteurs; c'est par là que plusieurs coccus présentent une thermorésistance exceptionnelle dans le lait, grâce à un capuchon de caséine que se forme autour d'eux par l'action de leur chymase (1915). Tout cela explique les difficultés de stérilisation du lait et indique la nécessité ainsi que les moyens de produire un lait de stérilisation facile (1937) en éliminant les germes thermorésistants et thermophiles; autrement le fait, apparemment paradoxal, se vérifie qu'un lait peut sortir du pasteurisateur plus riche en bactéries qu'il ne l'était avant la pasteurisation.

b. Air. Les lactocultures des acidoprotéolytes démontrent qu'en général l'aérobiose favorise la protéolyse, alors que l'anaérobiose favorise la saccharolyse (1897). À part cela les lactocultures permettent de reconnaître les différents besoins d'oxygène des bactéries selon qu'elles altèrent d'emblée toute la colonne de lait ou bien qu'elles commencent ou qu'elles se bornent à altérer le lait à sa surface ou dans le fond. Ces différences correspondent aux différentes manières par lesquelles s'accomplit la décoloration des lactocultures additionnées de tournesol, de bleu de méthylène etc. et expliquent les différentes manières de se com-

porter des bactéries suivant la hauteur du substrate, la largeur et la forme du récipient de culture, à l'appui de la distinction classique de BEIJERINCK en bactéries orthoaérophiles, microaérophiles et oligoaérophiles.

c. Nutrition. Les lactocultures donnent la preuve la plus éclatante, d'une part, de l'extrême sensibilité des bactéries à l'égard des conditions nutritives, d'autre part, de l'extrême complexité, délicatesse et inconstance de la composition ou, à mieux dire, de la constitution biophysico-chimique du lait, de sorte que ses propriétés nutritives et fermentatives sont susceptibles d'être modifiées même profondément et d'une manière même chimiquement indéfinissable, par des facteurs naturels ou artificiels. Il n'y a pas un lait mais des laits.

Quant aux modifications naturelles il me suffira de citer le lait que j'ai appelé disgénésique (1927), parce que, quoique normal en ce qui concerne les caractères organoleptiques et l'analyse chimique et micrographique ordinaire, il est impropre à la culture de ferments lactiques et acidoprotéolytiques.

Quant aux modifications artificielles il me suffira de citer celles que j'ai mises en rapport avec les différentes températures et les différents procédés de stérilisation thermique du lait (1917) qui le rendent plus ou moins favorable à la culture des bactéries saccharolytiques ou bien des bactéries protéolytiques.

Ces modifications peuvent être, pour la plupart, attribuées à la carence ou bien à la destruction d'un aliquid eugénésique, puisqu'elles sont ordinairement remédiables par l'addition de substances stimulantes ou de facteurs de croissance (peptone, extraits de viande ou de levures, sang, vitamines etc.) (1926).

D'ailleurs, les lactocultures sont bien favorables pour préciser la distinction des différentes vitamines et leur action sur le développement et sur les activités des bactéries; et d'autre part, les lactocultures ont révélé l'existence de principes microbicides ou inhibitoires ou contre-agissants qui sont propres au lait frais et qui sont plus ou moins thermostabiles.

4. MUTATION ET DISSOCIATION PHYSIOLOGIQUE DES BACTÉRIES.

Un autre avantage des lactocultures est de favoriser l'apparition de phénomènes de mutation (DE VRIES) c'est-à-dire de variation spontanée brusque et transmissible que j'ai appelé „mutation physiologique par divergences individuelles" (1921) et qui

se vérifie particulièrement par de petits ensemencements; à cette occasion j'ai donné aussi le premier exemple du procès de dissociation physiologique bactérienne, qui est devenu aujourd'hui un procès fondamental de bactériologie. L'aptitude spéciale du lait à provoquer la dissociation bactérienne a été reconnue ensuite par d'autres auteurs (Nungester (7)).

5. RAPPORT DE L'ACTION DES BACTÉRIES SUR LE LAIT AVEC LE SAPROPHYTISME ET LE PARASITISME.

Il y a des groupes bactériques (*B. typhi-coli*, *Staphylococcus*, *Streptococcus*) qui comprennent des espèces ou des souches dont la façon de se comporter dans le lait diffère d'un groupe à l'autre. D'après les auteurs certains groupes coagulent régulièrement le lait tandis que d'autres le coagulent inconstamment et même pas du tout. Or, notamment par la méthode de la culture sur gélose-lait, tous les types parviennent à coaguler, néanmoins par un mécanisme différent: les types acidifiants énergiques arrivent à coaguler par simple acidification, alors que les types faibles coagulent seulement par l'intermédiaire d'une chymase, par quoi ces derniers exigent ordinairement une observation plus prolongée. On observe en outre que le premier type se rencontre de préférence parmi les saprophytes, tandis que le second type se rencontre de préférence parmi les parasites; ce fait peut appuyer les recherches modernes concernant la relation entre l'activité protéolytique et la virulence des bactéries. Par conséquent les lactocultures peuvent rendre service à l'hygiène et à la pathologie comme moyen diagnostique auxiliaire pour déceler la nature et l'origine saprophytique ou parasitique des microbes (1923—1927).

6. FERMENTATION GAZEUSE ASSOCIATIVE.

Ainsi que je l'ai démontré (1933) le lait se prête tout particulièrement à l'étude du phénomène Nencki—Perfold de la fermentation gazeuse associative, qui dérive de l'action synergique de différentes bactéries dont chacune, à elle seule, est incapable de développer du gaz.

Conclusion.

En résumant: le lait comme milieu de culture est à même d'apporter une précieuse contribution à la connaissance de la physiologie, de l'enzymologie et du métabolisme des microbes, ainsi qu'à la connaissance intime du lait même, au service de la science pure et de la science ap-

pliquée, à la médecine, à l'industrie, à l'agriculture. Je n'ai pas besoin de rappeler la grande signification de la conquête de notions concernant la vie microbienne, aussi pour la biologie générale.

Pourtant, de tout ce qui précède découle la nécessité que l'étude de l'action des bactéries sur le lait soit fondée sur l'examen de plusieurs cultures faites dans des laits différents avec des ensemencements assez larges et sous des conditions différentes de l'ambiance; et il faut avant tout que l'observation soit faite avec l'oeil du biologiste et avec la diligence et la persévérance que Hugo de Vries pose comme conditions *sine qua non* pour ce genre de recherches.

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(From the Microbiological Laboratory of the Zuiderzee Reclamation Works, Medemblik).

THE INFLUENCE OF THE METHOD OF SAMPLING ON THE ACCURACY OF THE DETERMINATION OF BACTERIAL NUMBERS IN THE SOIL

by

G. W. HARMSEN

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1. INTRODUCTION.

Whereas much has been written about the accuracy and reliability of the plate-method and other methods for the counting of bacteria — see the list of references in the publication by HARMSEN and VERWEEL (1) — far less attention has been paid to the question of a possible unfavourable influence on the accuracy of counting bacteria caused by the way of sampling and the preparatory treatment of the samples. In fact, one is struck by the superficiality with which this side of the question is treated. Therefore, we deemed it desirable to obtain experimental data on the essential stages in the process of sampling and treatment of the samples.

2. METHODS.

We restricted ourselves to one counting method, *i.e.*, the plate-method, which we consider the most reliable and significant. This restriction was possible as with the dilution-method, the plate-method, or the direct counting of bacteria in stained microscopic preparations, the process of sampling and preparatory treatment of the samples is the same. We only determined the number of bacteria plus the actinomyces, as this group is known to make the highest demands on the counting.

Until recently our procedure for counting by the plate-method was the following.

The samples always consisted of a large number of borings, varying from 25 to 100, according to the size of the plot to be sampled. The

gimlets we used consist of a polished steel tube, 2.5 cm in diameter, with 1.2 mm thickness of the material. Over one third of their circumference is taken away. The rims and bottom ends are sharpened. At the top is a knob with a small handle for the pressing, turning, and drawing out of the borer. On the outside small marks have been made for regulating the depth of the sampling. When the borer is pulled out of the soil, the earth which protrudes from the open sides, is taken off with a flat knife along the sharp rim, as this part of the soil-cylinder has often been crumbled when the borer is pulled out. With a special flat spatule with round end, the often unreliable and crumbled-off bottom end of the boring is also removed, thus leaving only a smooth and regular part of the required length in the tube, to be shoved with the same spatule into a tight-fitting tin. In this way the large samples, often weighing several kg, are collected in the tins. These samples are then immediately taken to the laboratory, where on the very same day they are treated as follows.

The entire mass of earth is shed on a polished marmor plate, or, in the case of smaller samples, into a wide, flat enamelled dish, carefully crumbled by hands sterilized as much as possible with alcohol, and mixed or kneaded, when the samples are very wet and sticky. Meanwhile the whole mass is considerably reduced by successively throwing away half of it, while the remaining half is mixed more thoroughly and intensively again. Finally a quantity of some 200 g is left, which is specially pulverized once more and then spread out flat, whereafter the required small sample of exactly 5 g is weighed off. For this purpose a little earth is taken, at regular distances over the entire surface, with the point of a knife, and then put into a preweighed deep porcelan bowl. With a finger covered by a smooth rubber cup the small sample thus obtained is rubbed with sterile water or a sterile nutrient medium, until even the smallest lumps have disappeared, after which it is quantitatively washed in a small flask. Exactly 45 cc of medium is used for the diluting and washing, so that a suspension of 1 to 10 is obtained. Of this thick suspension a series of dilutions is made by repeatedly pipetting off 5 cc of a dilution and adding it to 45 cc sterile medium. The dilution ultimately required is then poured on ten parallel plates (for procedure see previous publication (1)). The utmost sterility is essential for all above manipulations, while the rubbing of the small sample, the preparation of the dilutions and the inoculating of the plates must be done under strictly aseptic conditions.

Thus the greatest possible reliability in the sampling and the preparatory treatment of the samples was aimed at, so that the latter could actually be considered as representative of the plots. Nevertheless, the results thus obtained were unsatisfactory, the parallel determinations often showing very marked fluctuations (fig. 1).

It is difficult to ascertain whether others may possess a more perfect

and more accurate method with better results, because the procedure of sampling and preparatory treatment of the samples is, as a rule, hardly described at all. Judging from their short statements, however, this does not seem likely, as this part of the process is generally treated most superficially; a very simple and inaccurate sampling seems to be considered as sufficient, which is quite contradictory to the care shown in the very process of counting. For example, it is often overlooked to suspend all clods and crumbs of earth, the sample being only shaken during a short time. This may suffice in the case of homogeneous, sandy soil without coarser organic particles, but most decidedly not when treating heavy soil. The number of borings per plot and the manner in which these are collected are also matters to which too little attention is paid; the samples are often simply collected with a spoon or spatule and even WAKSMAN (4), who has quite conscientiously worked out and described the methods for the counting of microbes, contents himself with mixing only three to four borings per sample.

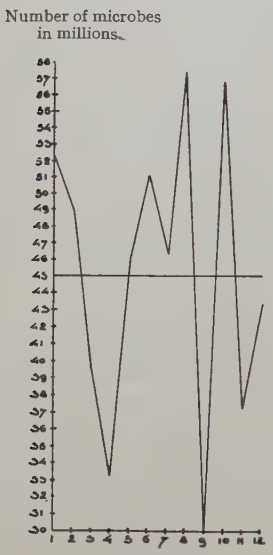

FIG. 1.

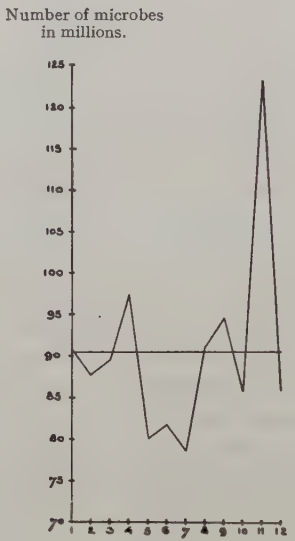

FIG. 2.

Though this superficiality was justified in the beginning, when the value of the counting as such still had to be ascertained, now that the importance as well as the measure of accuracy of the counting methods is sufficiently known and perfected, an equally accurate sampling

— without which the accuracy of the counting itself would lose most of its value — must be insisted on. We therefore thought it necessary to compare the accuracy of the sampling against the accuracy of the counting.

3. PRELIMINARY EXPERIMENTS.

In order to determine the value of each separate manipulation for the accuracy of the entire process of sampling, preparatory treatment of the samples, and counting, each manipulation in turn had to be left out, beginning with a complete counting on some plot or other.

For that purpose we chose a plot of 2 × 3 M used as arable land in the Wieringer-meer. We chose this very small plot with a view to reducing the possibility of unevenness of the soil and variations in the parallel determinations. All the same, a series of 12 samples of 25 borings each gave very marked fluctuations, far more than could be expected by the law of random samples. The mean deviation of the separate determinations (*i.e.*, the standard deviation) amounted to 21.7% of the mean, which is altogether far too high [1]) (See fig. 1).

This disappointing result is by no means accidental, for numberless previous standard deviation determinations had shown similar or worse results; from which we conclude that no better results can be obtained by our method of procedure, so that all countings made by us so far, will most likely be liable to these exeedingly high deviations. Moreover, the multiple countings made by other investigators showed equally pronounced variances. Accordingly, the determination of the microbes by the plate-method unfortunately remains very inaccurate when the method of sampling used so far by ourselves as well as others, is followed.

The first step we took in tracing the source of errors was eliminating the actual sampling, *i.e.*, the unevenness of the plot. We therefore took one more sample consisting of 25 borings from the same plot, and after the usual preparatory treatment, we took therefrom 12 portions of 5 g each, which were subsequently treated as separate samples. We thus got 12 countings from one and the same sample, but from 12 portions of 5 g that were separately weighed. The result did not show such

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[1]) The mean deviation of the separate determinations answers to the formula $\sigma_\alpha = \sqrt{\frac{\Sigma \alpha^2}{n-1}}$, α standing for the deviations from the mean M, and n for the number of the determinations in a series.

marked variations as in the case of the 12 separate samples, but yet the deviations remained far too high: the standard deviation being 13.0% of the average (see fig. 2).

The next step was eliminating the weighing of the 12 separate 5 g portions. So, after the usual preparatory treatment, we took only one portion of 5 g from a sample, and made it to a thick suspension of 1 : 10, whereafter 12 flasks were filled with this suspension diluted to 1 : 100, each of which then gradually diluted until a final dilution of 1 : 400.000 was obtained. We thus got 12 dilution series, giving 12 parallel results.

Now the picture was quite different: the 12 results showed comparatively small fluctuations, the standard deviation only amounting to 3.2% of the average (see fig. 3).

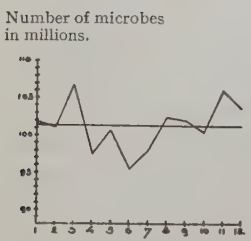

FIG. 3.

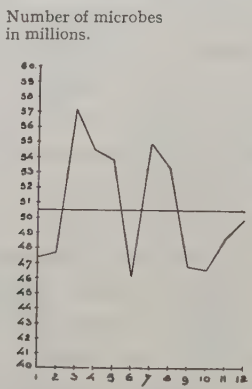

FIG. 4.

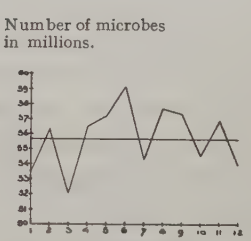

FIG. 5.

It was impossible to obtain a further improvement by eliminating the separate dilution series, only inoculating 12 series of ten plates each, of one final dilution; the fluctuations remained about the same. Moreover, a further improvement in the accuracy by eliminating special manipulations during the preparatory treatment, could hardly be expected any more, the accuracy of the last series already equalling the accuracy obtainable in one series of parallel plates, in other words, the accuracy of the actual plate-method. In fact the mean deviation — expressed in absolute measures — was 4.1, while the average of the 12 values for this deviation of the ten separate parallel plates in the 12 series, amounted to 13.3, from which $\frac{13.3}{\sqrt{10}} = 4.2$, which about equals

the mean deviation of the 12 separate series. The M-values of the 12 series, therefore do not vary more than those of arbitrarily collected groups of ten plates out of one series of 120 parallel plates.

These tests positively lead to the conclusion that the methods of sampling and preparatory treatment followed so far by ourselves and others, are not sufficiently accurate, which is, of course, also reflected in the results of the countings. In any case, the errors which thus occur during the process of sampling and preparatory treatment of the samples are far greater than the inevitable errors in counting. Accordingly, it is essential to improve the above manipulations before a further perfection of the actual counting technique will be of any use. This conclusion may be considered sufficiently well-founded, as the afore mentioned series have repeatedly given the same results.

4. THE INFLUENCE OF THE HOMOGENEISATION OF THE SAMPLE ON THE ACCURACY OF THE COUNT.

The weighing of the small portion for the suspension has thus proved to be the main cause of errors. The sampling in the field — even in case of a small plot of homogeneous soil — may also give rise to perceptible errors, seeing the greater variations shown by the 12 separate samples, as compared with those of the 12 portions weighed from one sample. Consequently, with plots of normal size, these errors adherent to the sampling may become quite considerable. The principal cause however, must be sought in the dishomogeneousness of the mixed sample, against which even the most careful mixing is of no avail. This would probably be of far less importance in case of a light, sandy soil, without larger remains of plants, but most soils remain without an intensive mechanic treatment so little homogeneous, that it will be impossible to weigh off two small portions that are actually similar.

An improvement in the accuracy in the microbial countings in the soil will have to be reached along the following lines: 1. the sampling of the plots will have to be carried out much more intensively. 2. the mixed sample obtained will have to be homogeneised as a whole, to be weighed off into the ultimate small portions afterwards.

The first exigency is much the hardest, and it will be well-nigh impossible to wholly meet it without rendering the counting process altogether too intricate and expensive. The above results plainly show that a mixed sample should consist of hundreds, perhaps even thousands, of borings to make the deviations between parallel samples smaller than those of the plate-method itself; and such intensive sampling

is practically infeasible. It will therefore be necessary to compromise between the desirable and the possible, and only carry the sampling to a maximum of perfection in case of the most accurate research.

The second requirement, on the other hand, is feasible without becoming too cumbersome. First of all we tried rubbing the soil through a fine sieve, or grinding it in a meatgrinder, a hammer-mill or a disc-mill. This, however, proved impossible with humid soil, in any case it required considerable extra work and loss of time. An easy homogeneisation of the soil in its natural state of humidity thus being out of the question, and in view of the impossibility to dry the soil before grinding — which would entail a considerable reduction in the number of bacberia — only one way was open to us, *i.e.*, making a suspension of the entire sample, often weighing several kgs. This could be comparatively quickly and easily done with the aid of a large porcelan ball-mill, in which the entire sample — after having been weighed — is rotated with a known quantity of water, or nutrient solution.

This method gave surprisingly good results. Even the heaviest and toughest soil, such as river-clay, or boulder-clay, and even soil containing hard pieces of peat or tough remains of plants, can be pulverized in this mill within the time of one hour, without leaving any perceptible or palpable grains; and in most cases half an hour suffices. A prolonged rotation may decimate the microbes, bruising and destroying too large a percentage of these, but one hour's or even two hours' rotation proved not to cause any noticeable reduction in their numbers. On the contrary, up to 1 to 1.5 hours' rotation — according to the type of soil — the microbes were found to have increased with the prolonged rotation time.

It goes without saying that the right filling of the mill, the rotation speed, the proportion of soil to water, the size of the balls, etc. (dependent on the kind of soil), will first have to be carefully determined, before the mill will give satisfactory results. But the proper judgment will come with some routine in these matters, and then failures can be easily avoided.

Unfortunately, however, the problem was not entirely solved by the use of this mill, as de-mixing occurred after the grinding, caused by the sedimentation of the larger particles, still leading to a rather pronounced variance in parallel determinations, especially of course in thin, liquid suspensions. The process of de-mixing is, of course, far slower in thicker suspensions, which renders a regular sampling much easier. But preparing viscous suspensions takes a lot of time, as the working

effect of the balls in the mill is much weaker when the grade of humidity is lower, which often necessitates a many hours' rotation to obtain an absolutely homogeneous suspension. However, once such a viscous suspension is obtained, the settling in it will be very slow, which makes it easy to take out small quantities with a little spade, which will give rather satisfactory tallying parallel determinations.

In the beginning we therefore made use of such thick suspensions. Fig. 4 shows a series of 12 parallel determinations made in the above way. The mean deviation of the separate determinations amounted to only 7.7% of the average, which compares favourably with the results shown in fig. 2, though the variance remains considerably greater than in the actual counting (compare fig. 4 with fig. 3) [1]).

So only using viscous suspensions was not quite satisfactory either, and not before having again proceeded to make thin, liquid suspensions, which we divided into small portions with a special apparatus, did we succeed in well-nigh eliminating the remaining variance.

This apparatus is a special kind of sectorial divider; the suspension flows through a fixed small funnel into a row of flasks placed on a horizontal, revolving disc, regularly passing under the mouth of the small funnel. We shall not give a detailed description of this apparatus, but only mention that it was not possible to pour the whole suspension into a larger funnel, and then, by opening a tap in the spout of that funnel, let the suspension flow evenly into the flasks, as this would have caused too much precipitation in such a large funnel. A really even division was only reached by siphoning the suspension from the revolving ball-mill directly into the flasks on the revolving disc. For that purpose we placed the mill in a sloping position, took off the cover, took the balls out of the suspension and let the mill rotate in this position, so that we could see the suspension rotating in it. We then carefully placed a thin glass tube in the deepest part of the slopingly rotating mill, taking care that the end of the tube just could not touch the mill. After a short but powerful sucking of the suspension, the latter was made to flow from the tube into the divider-apparatus placed under the mill, until the mill was quite empty. In this way one can be certain that the suspension is constantly and intensively mixed during the dividing process, so that an ideal division is obtained. Both the ball-

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[1]) All graphs are directly comparable, as being on the same scale; the distance from the mean to the x-axis (σ-value) is the same everywhere, *i.e.*, 10 cm.

mill and the divider-apparatus were worked by small electro-motors and could be easily adjusted to any number of revolutions desired.

By using a ball-mill, sterilised with alcohol, sterile flasks in the divider-apparatus and a sterile siphon with side-tube and clamp-tap, the entire manipulation can be accomplished without incurring any detrimental infection.

For a calculation of the dilution obtained it is necessary to know the proportion of the amount of soil to that of the solution in the mill. Weighed flasks must be used in the divider-apparatus for the determination of the quantity of suspension in each flask. It is then possible to calculate how much water or nutrient solution must be added from a burette to the suspension in the flask in order to obtain a suitable dilution from which the further dilutions can gradually be made.

The result thus obtained can be described as follows: Again one sample was taken from the above mentioned small plot of arable land and treated as before, but this time made into a thin, liquid suspension which, in the above described way, was siphoned from the rotating mill and divided into 12 portions by means of the divider-apparatus. The mean deviation of the separate determinations now only amounted to 3.65% of the mean (see fig. 5). So the variations ascertained in this series hardly exceed those of the plate-method. For comparison we would refer to fig. 3 and 5 and the characteristics of the respective series. We had thus succeeded in perfecting the preparatory treatment of the samples to such an extent, that its variations were hardly greater than those inherent to the other stages of the counting of bacteria. The still remaining slight increase in the variance was of so little importance that further efforts towards reaching a greater perfection of the homogeneisation and dividing process did not seem justified.

Owing to the above improvements, the preparatory treatment of the soil samples can be considered as accurate as the counting itself, which makes it possible to successfully aim at a further perfecting of the plate-method; for in the procedure followed hitherto, the crude preparation of the samples has clearly been the limiting factor.

5. THE INFLUENCE OF THE HOMOGENEISATION OF THE SAMPLE ON THE ACCURACY OF THE DETERMINATION OF SOLID MATERIALS IN SOIL.

While the above has shown the importance of a perfection in sampling and preparatory treatment of the samples in determining the number of bacteria, the advantages of this procedure will not be most appa-

rent in this determination with her still comparatively poor accuracy. The determination of various chemical and physical properties on the other hand can be made with a far greater measure of accuracy. Therefore the weak points in the sampling and the preparatory treatment of the samples can herewith be more easily ascertained. For this reason we decided to trace the effect of the improved treatment of the samples by the determination of some of these properties.

On the basis of their distribution in the soil, substances of importance for soil investigations can on the whole be divided as follows:

Group 1. Substances dissolved in the moisture of the soil, the concentration of which is constant throughout the sample.

Group 2. Liquid or gaseous substances, still mobile, and with a tendency towards a regular spreading throughout the soil.

Group 3. Quite immobile, bound, solid substances, incapable of eliminating a discontinuity, occurring in their distribution.

Group 4. Living organisms or their products, which not only let a prevailing discontinuity exist but even activily give rise to it.

To the fourth group belong a.o. the micro-organisms whose formation of colonies causes local accumulations, which again result in a pronounced micro-dishomogeneousness, so that even adjacent soil grains are often found to show great differences in bacterial numbers.

This group certainly makes the highest demands on the preparatory treatment of the samples and the sampling, and would most readily respond to a perfected treatment, were it not that the accuracy of the determination itself is so slight.

The group of solid substances (group 3) is the one showing most resemblance to the living organisms. We chose calcium carbonate as an example, as this can be comparatively easily and quickly determined. The gas-volumetrical methods according to SCHEIBLER or PASSON, generally used for mass research, being too crude for our purpose, we applied the classic method, *i.e.*, the substance to be tested was boiled in diluted hydrochloric acid in a current of CO_2-free air, and the carbon dioxide thus developed was caught into U-tubes with soda-lime. This method of analysis is very accurate, as is shown in fig. 6, giving the percentage of $CaCO_3$ in a sample of $CaCO_3$ (pro analysi Kahlbaum), made in twelve-fold. The mean deviation of the separate determinations here only amounts to 0.2% of the mean.

FIG. 6.

In the 12 parallel soil samples, taken as previously described, from the same small plot, the $CaCO_3$ percentage was found to vary as indicated in fig. 7. The mean deviation of the separate determinations proved to be 7.3% of the mean, which variability actually is much smaller than in the case of the counting of bacteria, though the variance remains quite considerable.

Further tests, moreover, showed that in this case, more so than in that of the counting of bacteria, the micro-dishomogeneousness was the main cause of the variance, which is found in the weighing of the portions of 1 to 2 g finally to be analysed, whereas the samples themselves do not show such great mutual differences. 12 portions taken from one sample were found, when analysed, to have almost the same σ_α, *i.e.*, 7.4% of M (see fig. 8). On the other hand, the variance between the 12 separate samples can be strongly reduced, if these are dried and ground before the portions are taken for the analysis. In this way we obtained a series with a σ_α of only 4.4% of M (see fig. 9). This is explained by the fact that the calcium carbonate is, to a great extent, present in the soil in the form of rather coarse shel fragments, and the portions only weigh 1 to 2 g instead of 5 g.

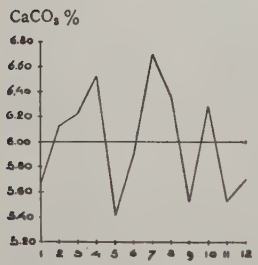

Fig. 7.

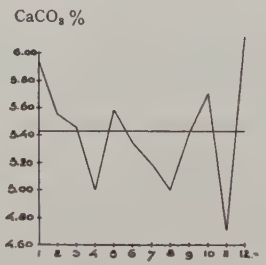

Fig. 8.

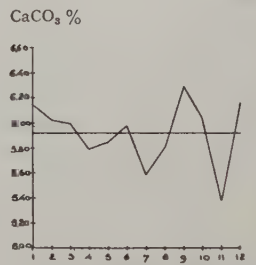

Fig. 9.

The 12 separate samples, however, yet showed some mutual variability, for 12 portions taken from o n e of the dried and ground samples, still gave a much better series, with $\sigma_\alpha = 1.55\%$ of M, in other words, a fairly tallying value (see fig. 10). And the accuracy of this determination in dried and ground samples could still be further perfected by a finer grinding of the soil.

So far we used the ordinary laboratory disc-mill, which, however, grinds rather irregularly and not fine enough. By using a rapidly revolving laboratory grinding-mill of Messrs. Pepping of Amsterdam,

with sieve-holes of 0.5 mm diameter, we were able to obtain the series indicated in fig. 11, with $\sigma_\alpha = 1.15\%$ of M, and by making use of the same mill with sieve-holes of 0.25 mm, the variance was even reduced to $\sigma_\alpha = 0.57\%$ of M (see fig. 12). This result fairly approaches the possible limit, *i.e.*, the accuracy of the $CaCO_3$ analysis itself. Some improvement might have been obtained with a still finer sieve, but the too finely ground soil would then have become too difficult to handle, so that for technical reasons it does not seem advisable to further continue in this direction.

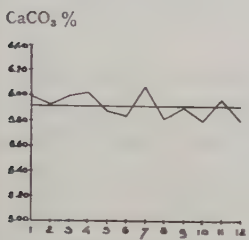

FIG. 10.

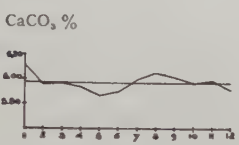

FIG. 11.

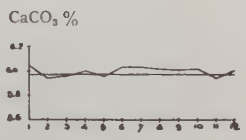

FIG. 12.

Using a sectorial divider-apparatus for the dry samples, where we at first also feared a very rapid de-mixing, proved fruitless. We made use of this apparatus in repeating the test-series under fig. 10. The results most certainly were no better than those reproduced in fig. 10; as can be seen from the curve in fig. 13 with $\sigma_\alpha = 1.65\%$ of M. In soil samples, all particles of which have about the same specific weight, the risk of de-mixing is not very great, only in case of complex substances, with strongly varying specific weights and very differently shaped particles, it will be necessary to take measures against de-mixing.

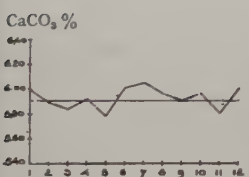

FIG. 13.

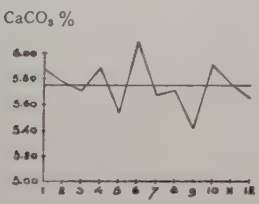

FIG. 14.

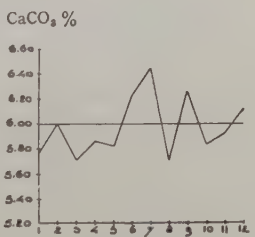

FIG. 15.

The homogeneisation of wet samples in a ball mill gave a less satisfactory result than the drying and grinding process. The variance in

the series obtained was considerably greater. A thick, mashy suspension gave the series reproduced in fig. 14 with $\sigma_\alpha = 3.1\%$ of M, while a thin, liquid suspension, after division by means of the sectorial divider-apparatus, gave an even more unfavourable picture with $\sigma_\alpha = 4.0\%$ of M (fig. 15), presumably as a consequence of the rapid settling of the coarser shell-grit parts.

The homogeneisation of wet samples in a ball mill thus proves to be decidedly inferior to the normal grinding of dried samples. For this reason we would not recommend this mill for most determinations, exept when the samples can not be dried, a.o. for the determination of the number of bacteria, when this mill is a very useful substitute for the ordinary grinding of the dry samples.

6. THE INFLUENCE OF THE HOMOGENEISATION OF THE SAMPLE ON THE ACCURACY OF THE DETERMINATION OF THE WATER CONTENT.

As representative of the second category of substances *i.e.*, of the mobile substances in the soil, we chose the water content of same. The division of the moisture in the soil proved to be much more regular than that of calcium carbonate or of bacteria. 12 parallel samples of the same plot, taken in the usual way, gave the series shown in fig. 16 with $\sigma_\alpha = 1.75\%$ of M.

This insignificant variability was due to two causes, *viz.*, the mutual variability of the samples, and the micro-dishomogeneousness of the soil in one sample.

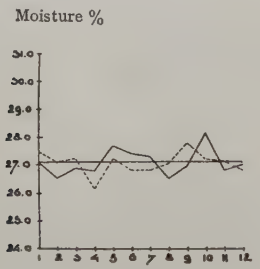

FIG. 16.

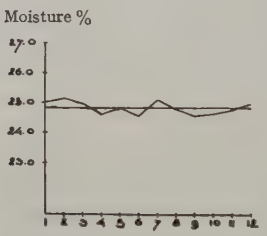

FIG. 18.

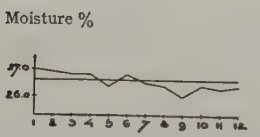

FIG. 17.

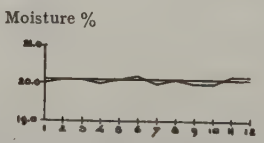

FIG. 19.

The variance caused by the sampling was found to be quite small, though clearly perceptible, for 12 portions taken from one sample gave a series with the appreciably smaller value of $\sigma_\alpha = 1.15\%$ of M (see fig. 17). The remaining variability again proved to be caused by the micro-dishomogeneousness of the soil: from one sample, homogeneised in the ball mill to a mashy thick suspension, we took a series of 12 portions, which mutually differed very little which $\sigma_\alpha = 0.8\%$ of M (see fig. 18). And an analogous series which was obtained with the aid of the divider-apparatus from a thinly-liquid suspension, even gave $\sigma_\alpha = 0.4\%$ of M (see fig. 19).

The accuracy of the moisture determination consequently could be noticeably perfected by a proper homogeneisation in the ball mill. Naturally it was impossible in this case to dry the samples by way of preparatory treatment.

It will therefore be decidedly advisable for an accurate determination of the moisture content, to homogeneise the samples in a ball mill. Likewise it will be of great importance to perfect the technique of sampling as much as possible, because even in the case of the extremely homogeneous plot of arable land that was tested, the 12 separate samples showed a considerably greater variance than the 12 portions taken from one mixed sample.

7. THE INFLUENCE OF THE HOMOGENEISATION OF THE SAMPLE ON THE ACCURACY OF THE DETERMINATION OF DISSOLVED SUBSTANCES.

We finally had to include in our experiments a substance dissolved in the moisture of the soil (group 1), as such a substance, independent of the structure of the soil, will always show a tendency towards a continuous distribution throughout the water in the soil. We chose the Cl-ion, for the easy determination of which we were fully equipped. The Cl-percentage of the moisture in the soil is no independent value and can only be determined in case of a simultaneous determination of the moisture content. Both determinations are always made in the same portion, so that only the very slight variability of the moisture determination itself could be misleading when judging the variability of the Cl-determination; not so, however, the inaccuracy of the moisture determination caused by any unevenness of the soil.

Unfortunately, the plot used so far proved unsuitable for the determination of the Cl-percentage, because the soil contained hardly any salt at all. So we had to chose a plot rich in salt situated on the grass-

land outside of the dike on the ,,Balgzand", North of Ewijcksluis. For this reason the variability of the Cl-determination is not altogether comparable with the variability of the other substances discussed in this paper, the more so as, so far we had to do with very homogeneous soil (owing to repeated treatment as arable land), while this was an untreated sandy ,,gras-schor" with a compact Puccinellia-turf, and distinctly layered. The variability of the 12 parallel samples — again taken in the usual way within an area of 3 × 2 M — was here considerably greater than in the case of the moisture determination on 12 parallel samples taken from the previous plot.

We found the series reproduced in fig. 20 with $\sigma_\alpha = 3.95\%$ of M. This variability, however, must principally be accounted for by the considerable irregularity of the soil in question, with pronounced fluctuations of the Cl-ion concentration in the various layers, and not by the micro-dishomogeneousness of the Cl-concentration between small particles of soil. A series of 12 portions from one mixed sample,

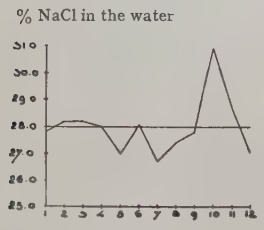

FIG. 20.

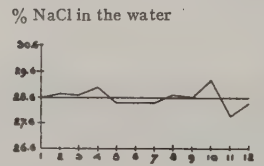

FIG. 21.

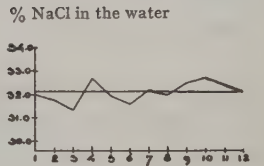

FIG. 22.

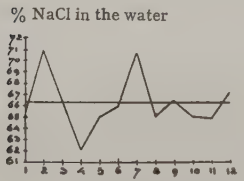

FIG. 23.

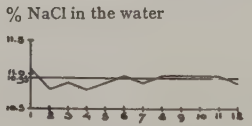

FIG. 24.

in fact, had a very small value of $\sigma_\alpha = 1.25\%$ of M (fig. 21). At the same time this proved to be the highest grade of accuracy obtainable in salt determinations in the soil, for a homogeneisation of the sample in the ball mill repeatedly gave — both with very thick mashy, as with thin, liquid suspensions — σ_α = ca. 1.25% of M (fig. 22). Moreover, homoge-

neisation by drying and grinding gave worse series, with $\sigma_\alpha = 3.75\%$ of M (fig. 23). This is explained by the fact that the salt crystallizes during the drying of the samples, localizing itself in the form of crystals on certain particles of the soil. In other words, as regards the spreading of the NaCl, the sample is strongly de-mixed owing the drying process.

Quite as expected, we found that with substances of group 1, which in normal circumstances are entirely dissolved in the moisture of the soil, an intensive homogeneisation of the samples is superfluous, as the concentration of this moisture throughout the soil is well-nigh the same. The parallel determinations made in portions weighed off without any special care from an indifferently mixed sample, tally fairly well (fig. 21) and come pretty near the optimal measure of accuracy that can be reached in titrimetrical Cl-determinations. These were arrived at by making 12 determinations in one sample of soil extract (see fig. 24 with $\sigma_\alpha = 0.84\%$ of M).

In conclusion, we can in the first place state that the above described series-determinations have demonstrated the necessity to homogeneise the large mixed samples taken in the field, in order to counterbalance the micro-dishomogeneousness in the division of the factor to be determined. This is most clearly shown in the case of solid substances and living organisms in the soil, with the discontinuously divided entities of groups 3 and 4; and less clear with the liquid or gaseous substances of group 2; while with the substances dissolved in the moisture of the soil there is no question of micro-dishomogeneousness.

The second conclusion arrived at is, however, that the perfecting of the sampling — increasing the number of borings from which the mixed sample is obtained, and the very accurate and exact manner in which these borings are executed — in order to eliminate the macro-dishomogeneousness in the plot to be sampled must always be considered of vital importance for the accuracy and reproductiveness to be reached in the determination. Consequently it is no superfluous luxury to make hundreds of borings for one sample in the case of large plots. That such intensive sampling is bound to certain limits on account of the available time, labour and cost, is inevitable; but it is certainly possible and essential to pay more attention to this question in future.

With respect to the live and solid substances, an intensive homogeneisation of the samples is a matter of still greater urgency than an improvement in the sampling. Without the first, even the best sampling-method and the most perfected analysis lose their value.

All these determinations have again shown how difficult it is to make soil analysis. The very complicated structure of the soil entails certain difficulties that do not exist with other substances, such as water, milk, various food-stuffs, etc.

8. THE INVESTIGATIONS OF THORNTON AND TAYLOR.

Though for a long time we had felt that it was desirable to study the influence of the method of sampling and the preparatory treatment of the samples on the accuracy of the counting of bacteria, a lecture held by THORNTON and TAYLOR at the 3rd Congress of the International Society of Soil Science at Oxford in 1935 (3), gave the decisive impuls to the above tests. THORNTON and TAYLOR think they have ascertained very strong fluctuations in the numbers of bacteria determined in the soil, within the time of one or merely few hours. During two days they de-

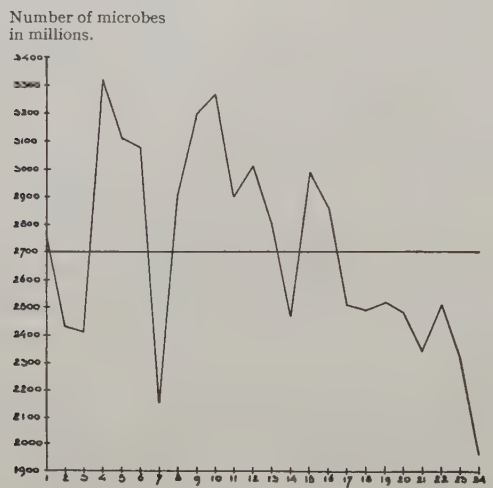

FIG. 25.

termined the bacterial numbers in the soil of a small and homogeneous plot of arable land, both according to the plate-method, as by means of direct countings in stained preparations of soil suspensions, with intervals of only one hour. As a matter of fact, the figures they arrived at varied quite considerably, and the σ_α-values in the series published by them are very high indeed: the direct counting giving $\sigma_\alpha = 13.6\%$ of M, and the plate counting $\sigma_\alpha = 17.5\%$ of M (fig. 25 and 26); and that while the points of the 2 graphs only represented the averages of

two adjacent plots. In the case of one plot the fluctuations were even greater, thus of the same order as the series obtained by us (fig. 1).

THORNTON and TAYLOR conclude from these figures that very considerable fluctuations in the number of microbes in the soil actually occur within one or more hours. They therefore talk of „short-period-fluctuations". They express the opinion that these fluctuations are

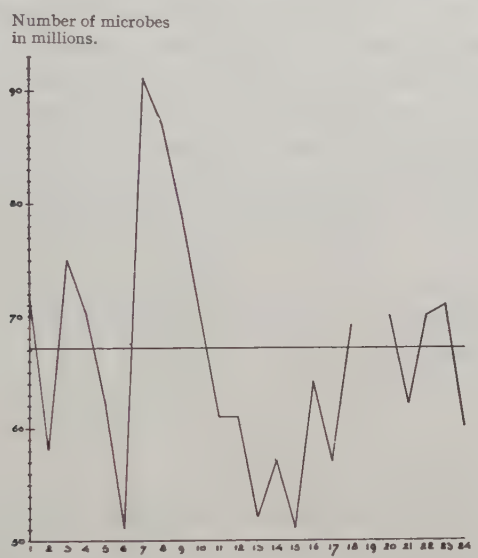

FIG. 26.

absolutely real, without explaining in how far the figures arrived at by them are accurate and reliable. About this point the reader is mostly left in the dark, as the methods of sampling, of the preparatory treatment of the samples, and of the counting itself, are hardly described at all. Their sole argument in refusing any justifiable doubts as to the accuracy of the figures in question is that they devided the plot in two halves and sampled both halves separately, so that they got two parallel results.

These duplicate results now show parallel fluctuations at large, which gives the impression that these are not due to an accidental variability of the determination, but that they are real and founded on simultaneously occurring ups and downs in the bacterial population. This, however, need not be the case at all, as inconstancy in procedure may also lead to such fluctuations. The only thing that follows from this

parallelism is that these appreciable fluctuations may not only be caused by the sampling and the preparatory treatment of the samples, but also by the methodical inconstancy during the counting. This inconstancy, however, will be less accentuated when a standardisation of the counting technique is consistently carried through (see series in fig. 3). On the other hand, the discreapancys often found between the determinations made in the two half plots, are presumably due to an inadequate sampling and preparatory treatment of the samples.

The conclusion arrived at by THORNTON and TAYLOR loses much of its probability as the fluctuations in the absolute numbers of bacteria found by direct counting, are not in correspondingly greater measure reflected in the results obtained by means of the plate-counting. An increase in the absolute number of microbes with approximately 10% within an hour, as repeatedly occurs in the series of THORNTON and TAYLOR, after all means an increase of 100% and more in the number of young, active bacteria! And of this the plate-counting shows nothing at all. On the contrary, the ups and downs of the direct counting are by no means parallel with those ascertained at the plate-counting. So there is no question whatever of some correlation between the two series.

A second refutation of the conclusion of THORNTON and TAYLOR is the following: every increase in the absolute number of microbes — if real — means an actual formation of new microbes, so that all increases during one day may be totalized for determining the minimum of newly formed microbes for that day. How to explain the depressions between these increases, *i.e.*, the very rapid loss of so many microbes, remains a difficult question in itself. However, leaving this improbability entirely aside for the moment, the fact remains that the total of new increases in microbes as shown in the series of THORNTON and TAYLOR is incredibly high. Besides, it decidedly represents a *minimum*, for even in periods when these numbers show a decrease, or remain constant, new microbes may be formed apart from the loss of others. From fig. 25 a production of abt. 3×10^9 microbes per day and per gram of soil may be derived. Taking as a basis an average volume per microbe of abt. $1/3\,\mu^3$, ca. $10^9\,\mu^3$ of microbe mass per gram of soil would be formed per day, *i.e.*, abt. 1 mm^3, and per year ca. 360 mm^3, or more than $1/3\,cm^3$. This value in itself seems incredibly high, but it becomes quite absurd in view of the fact that the formation of young microbes can only take place at the cost of organic material, a small percentage of which, at the most, can regenerate in the form of microbes. This would mean

that several cc.'s of organic matter would be decomposed per cc of soil per year. This now is quite decidely impossible.

It immediately follows from the above that the fluctuations given in the series of THORNTON and TAYLOR are not real, and for the greater part founded on methodical errors. Yet we thought it advisable to repeat their test. We therefore took another series of 12 samples from the plot repeatedly used for the purpose, in the same way as before, but now with intervals of two hours each, so that we got a regular sampling during one full space of 24 hours. These 12 samples were treated meticulously alike, though they were not homogeneised, but pulverized by hand, and mixed according to our old method (see description on page 179). They gave the series reproduced in fig. 27, with $\sigma_\alpha = 14\%$ of M,

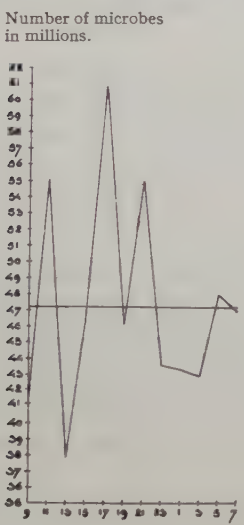

FIG. 27.

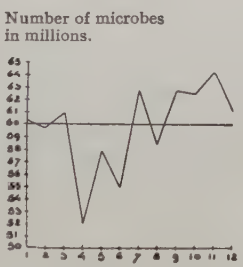

FIG. 28.

which by no means compares unfavourably with the series of 12 samples from the same plot, simultaneously taken and similarly treated, as shown in fig. 1. There are no indications whatever of a higher variability; we would rather say the contrary.

This in itself was a refutation of the theory of ,,short-period-fluctuations''. However, to make quite sure, we repeated the test once more, following the improved method of procedure, after homogeneising the whole sample in the ball mill. We again took samples from the same plot, with intervals of 2 hours during a full 24 hours, and treated these by homogeneising them each time as a whole in the ball mill. The series

thus obtained completely confirmed our assumption that in our case there was no question at all of the marked and very rapid „short-period-fluctuations" in the number of microbes in the soil, ascertained by THORNTON and TAYLOR. The curve in fig. 28 shows great similarity with the graph reproduced in fig. 5 of the series of 12 samples simultaneously taken and inoculated in the same way after being homogeneised. Also the value of $\sigma_\alpha = 5.7\%$ of M is quite of the same order.

9. THE INVESTIGATIONS OF JAMES AND SUTHERLAND.

Exactly at the time when the above described tests were almost completed, JAMES and SUTHERLAND published two articles on the same subject (2). The writers arrived at the same conclusion as we did: that perfecting the counting technique is no good at all, so long as the preparatory treatment of the samples and the sampling give rise to so much greater errors.

They even mention a third source of errors besides the two to which we have called attention. In their opinion not only the taking of samples in the field and the weighing of the small samples taken from the large mixed sample may lead to errors, but also the preparation of the dilutions of the small sample. Our tests showed, however, that this third source did not exist, which is presumably due to the fact that the burettes and pipettes used by us for the preparation of these dilutions, were gauged far more accurately than those of JAMES and SUTHERLAND. Moreover, the flasks with sterile water were not sterilized by us after the filling, but the sterilized water was poured from a burette into sterile flasks. Owing to these precautions, any appreciable variance caused by the inaccuracies of the dilution process can be absolutely avoided.

The above workers, however, do not try to fight the marked variance resulting from the micro-dishomogeneousness of the soil in a large mixed sample, technically, like we did by homogeneising the entire sample, but statistically, by not weighing off one portion from the large sample, but several portions, determining the number of microbes in each of these, and calculating the average of the results obtained. This eliminates the ball mill and the divider-apparatus, but multiplies the amount of work.

For very accurate determinations a combination of the proposition of JAMES and SUTHERLAND and ours might even be feasible. Thus the last remaining undesirable variance of our method could be eliminated by weighing off several parallel portions from a homogeneised mixed sample.

Summary.

Tests were made to determine the influence of the sampling and the preparatory treatment of the samples on the counting of bacteria in the soil. These proved that the present methods are very inaccurate and give rise to considerable variance in parallel determinations, to the extent even of rendering the effect of a perfected counting procedure quite negligible.

In this connection it has been stated that it is useless to aim at a minute counting of bacteria, as only the number of microbes in the ultimate soil suspension would thus be determined, whereas this suspension is by no means accurately representative of the tested soil.

For a better preparatory treatment of the samples a homogeneisation method by means of a porcelan ball mill was worked out, according to which a suspension is made of the sample with water, which method has given satisfactory results.

Finally, the determination of various chemical substances, apart from the counting of microbes, has been included in our tests. Here also an improvement in the treatment of the samples was arrived at.

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(From the Division of Plant Nutrition, University of California, Berkeley, California).

STUDIES UPON THE METHANE FERMENTATION. IV. THE ISOLATION AND CULTURE OF *METHANOBACTERIUM OMELIANSKII*

by

H. A. BARKER

(Received May 7, 1940).

One of the most interesting of the methane-producing bacteria is *Methanobacterium Omelianskii* (1). This organism, first observed by OMELIANSKI, causes an incomplete oxidation of organic compounds. Ethanol, for example, is oxidized only as far as acetic acid. It is this limited oxidizing ability which made possible the demonstration (2) that the methane produced in the fermentation of organic compounds is formed by a reduction of carbon dioxide in accordance with the general equation:

$$4\,H_2A + CO_2 \rightarrow 4\,A + CH_4 + 2H_2O \qquad (1)$$

This organism is also interesting because in association with a hitherto undescribed anaerobic spore former [1]) it brings about the conversion of ethanol into a mixture of caproic, butyric and acetic acids (3). And further, *Mb. Omelianskii* appears to be one of the most abundant bacteria active in the anaerobic digestion of sewage sludge (4).

All previous work upon *Mb. Omelianskii* was carried out with impure enrichment cultures because pure cultures had not been isolated. Although this circumstance need not invalidate the conclusions drawn from cultural and biochemical experiments with the organism, still it introduces an element of uncertainty in their interpretation. Furthermore there are many additional problems connected with the morphology and physiology of these bacteria that cannot be successfully undertaken without the use of strictly pure culture. For these reasons the isolation and pure culture study of *Mb. Omelianskii* were undertaken.

[1]) A description of this organism will be published in the near future.

1. ISOLATION OF PURE CULTURES.

Enrichment cultures for *Mb. Omelianskii* were obtained by inoculating fresh water or marine muds into a medium [1]) containing C_2H_5OH and $CaCO_3$ as the principal ingredients (1). The cultures were incubated anaerobically at 35—40° C. After a few days an active methane fermentation began, the alcohol being converted to acetic acid. After several successive transfers in the same medium, a series of agar shake cultures (medium A) was inoculated in seven dilutions as previously described (1). The agar in the tubes inoculated with the first four dilutions was split by gas within one week. Bacterial development in the higher dilutions occurred more slowly. The seventh dilution did not show evidence of gas production until the 33rd day. This tube was then immediately opened and found to contain one large lens-shaped colony (about 1 mm diameter) and a number of smaller (0.1—0.25 mm diameter) colonies.

Fig. 1. Large soft colony of *Mb. Omelianskii* in agar. Smaller diffuse colonies are visible at the tip of the large colony and at the lower right (white blur). Surrounding the large colony there are a number of small hard colonies (small white spots, slightly out of focus). Unstained. × 46.

The large colony and three of the smaller colonies were used to inoculate four new series of agar shake cultures. Nothing grew from the smaller colonies, while the tubes inoculated from the large colony developed through the third dilution.

All these tubes of the second series contained three types of colonies. The largest (1—2 mm diameter) and most conspicuous were compact, more or less lens-shaped colonies of a soft consistency and a light yellowish brown color (Fig. 1). These were like the colony used for

[1]) Composition: tapwater, C_2H_5OH 1%, $CaCO_3$ 10%, NH_4Cl 0.05%, K_2HPO_4 0.1%, $MgCl_2$ 0.01%, pH 7.4. To transfers there were added per 100 cc: 2 cc of a 1% $Na_2S \cdot 9H_2O$ solution and 5 cc of a 5% Na_2CO_3 solution.

inoculating this series of tubes. The other two colony types were considerably smaller than the first. One was compact (occasionally with a dendritic fringe) and hard, *i.e.*, not readily broken up into its component cells (Fig. 2). The other colony type was very diffuse in structure, being reminiscent of an actinomyces colony (Figs. 3 and 4).

Fig. 2. Two small hard colonies in agar. Erythrosine. × 320.

The cells making up both of the smaller colony types were difficult to remove from the agar, one because of its hardness, the other because of its diffuse structure. The contents of the large soft colonies on the contrary could easily be drawn up into a capillary pipette for examination or inoculation. The significance of

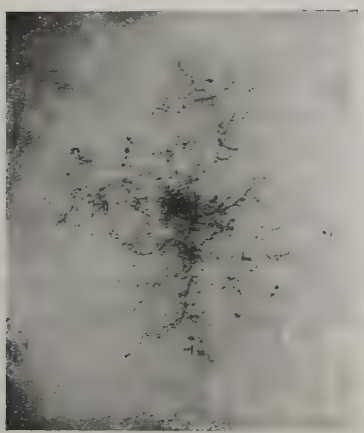

Fig. 3. Diffuse type of colony in agar (not crushed). Erythrosine. × 145.

Fig. 4. Portion of small hard colony (slightly crushed) and of a diffuse colony (main part deeper in agar), showing cells of both. Erythrosine. × 350.

these three colony types will be discussed later. The cells in all three colony types were essentially the same in appearance, being rather small, apparently non-sporeforming rods such as were previously described for *Mb. Omelianskii* (1).

From the third dilution of the second series of shake cultures, six

single colonies were inoculated into separate tubes of semisolid agar medium (medium A). These colonies were all of the large soft type. Within nine days bacterial development became visible in all six tubes. Also gas was produced and the medium became increasingly acid. The acidity was shown to be due to the formation of acetic acid. The bacteria were therefore physiologically as well as morphologically of the *Mb. Omelianskii* type.

Of the six strains so obtained, one (strain 2) was used for all of the tests and experiments to be described. It may be mentioned that the other five strains and also two additional strains isolated independently by similar methods, were not observed to differ in any respect from strain 2. They were, however, not studied in nearly such great detail.

Evidence of purity. The first question that arises is that of the purity of the cultures so isolated.

The possibility of the presence of aerobic contaminants was first investigated by heavily inoculating yeast-dextrose-agar plates which were incubated at 28° and 37° C. for a week. An ethanol-ammonium nitrogen-agar medium was also used. When no colonies developed on either medium it was concluded that the cultures were free of aerobic contaminants.

Because of the strict anaerobiosis maintained in the enrichment and isolation cultures, aerobic bacteria could, however, hardly be expected as contaminants. The presence of obligate or facultative anaerobes would be much more probable. To detect these stab cultures were made into an agar medium containing yeast extract and glucose. The tubes were sealed and made anaerobic with a pyrogallol-K_2CO_3 mixture. No organisms developed from any of the cultures tested after 1—2 weeks incubation at 37°C.

Contamination by aerobic and the ordinary sugar and amino acid fermenting anaerobic organisms was thus made highly improbable. During subsequent experiments on the physiology of the methane bacteria, media containing a wide variety of organic compounds were inoculated and incubated under strictly anaerobic conditions for periods of 1—4 weeks. In none of these media (except in a few instances where obvious contamination occurred) was there any macroscopic or microscopic evidence of the presence of organisms other than the methane bacteria. Whenever growth occurred methane was formed and the same morphological types of cells described below were observed. There was no evidence of any separation of forms such as would be expected with a mixed culture.

On the basis of the above evidence it may reasonably be concluded that strain 2 is indeed pure, though the possibility is not excluded that the culture represents a mixture of physiologically similar but morphologically somewhat distinct organisms. This possibility will be next considered.

It has already been mentioned that during the isolation of strain 2, three colony types appeared in the second series of shake cultures which had been inoculated from a single, well isolated and apparently pure colony. Subsequently, shake cultures inoculated with strain 2 were repeatedly observed to contain the same three colony types. The question therefore arose as to whether we were dealing with three distinct species or with three variants of a single species.

In order to answer this question it was decided to see whether by repeated reisolation a culture showing only one type of colony could be obtained. Because of the greater ease of transferring the large soft colonies, an effort was first made to separate this colony type from the two smaller types.

The original culture in semisolid agar was first inoculated into a series of agar shake cultures in seven successive dilutions. During the course of several weeks incubation gas was produced in all tubes through the fifth dilution; as was expected, these tubes contained the three colony types, already described. Most abundant, though least conspicuous, were the small hard colonies. The diffuse colonies were somewhat less numerous, while the larger soft colonies were still much less numerous. The ratio of the small hard and the diffuse colonies together to the large soft colonies appeared to be of the order of 10 : 1 to 100 : 1.

The fifth dilution tube contained a single apparently well isolated large soft colony in addition to several small colonies of the other types distributed throughout the tube. So far as could be ascertained there were no bacteria growing between the agar and the glass. The tube was opened, the section of agar containing the large soft colony was cut out and the contents of the colony were removed by being sucked into a sterile capillary pipette. The cells so obtained were inoculated into agar shake cultures in four successive dilutions (each about 12 : 1). Within 13 days colonies were visible and gas had been produced in all four tubes. Now since the inoculating colony could not possibly have been more than very slightly contaminated with cells from colonies of other types (and probably was not at all contaminated), one would expect the colonies in the new series of shake cultures to be predominantly of the

large soft type. This expectation was not fulfilled. In all four dilutions the small hard and the diffuse colonies were again 10—100 times as numerous as the large soft colonies. This could not only mean that all three colony types developed from cells present in the large soft colony of the previous series. This conclusion was further supported by making a third series of shake cultures inoculated with single large soft colonies of the second series. The new tubes again contained tens of thousands of small hard and diffuse colonies. The ratio of these types to the large soft type being at least 1000 : 1 in some tubes. Intermediate types between the large soft and small hard colonies were observed here as elsewhere.

Since bacteria forming the large soft colonies could not be separated from those forming the other colony types, an attempt was made to separate the latter from the former. Experiments in this direction were not entirely satisfactory because it was impossible to remove the small hard and the diffuse colonies from the agar in such a way as to make possible solid medium to solid medium transfers. Recourse was therefore had to solid-to-liquid-to-solid medium transfers which are never entirely satisfactory for purposes of isolation since the partial purification achieved in the solid medium may be entirely lost in the subsequent liquid culture.

The attempt to separate organisms forming diffuse colonies was carried out as follows: A culture of strain 2 was inoculated into a series of agar shake cultures in 8 dilutions. Gas developed in the tubes of the first six dilutions within 9 days; these tubes contained all three colony types. At the end of 19 days' incubation the seventh tube had not yet shown evidence of gas production (splitting of agar) but it could be seen to contain about 10 diffuse and small hard colonies. There were no large soft colonies. The tube was opened and four isolated diffuse colonies were cut out of the agar and transferred to separate tubes of semisolid agar. Within six days all four tubes showed growth and gas formation. After abundant growth had occurred three of these tubes were used to inoculate three series of shake cultures in five dilutions. All three series developed colonies of the three types to the highest dilution. As in all previous cultures the small hard colonies were most abundant, the large soft colonies least abundant. This again indicated that the different colony types are formed by variants of a single pure culture and not by three distinct organisms.

2. MORPHOLOGICAL AND STAINING CHARACTERISTICS.

The cells of *Mb. Omelianskii* are thin unbranched rods which may be either straight or bent (Fig. 5). The length varies from 1.5 μ to more than 10 μ. The most common length is 3—6 μ but filamentous forms (10 μ or longer) are also quite abundant in many cultures. The width is 0.6—0.7 μ. There are no consistent differences among the cells of the three colony types, but generally the cells of the diffuse colonies tend to be longer, more bent and slightly thinner than the cells of the other colonies. Some of these differences are shown in Fig. 4.

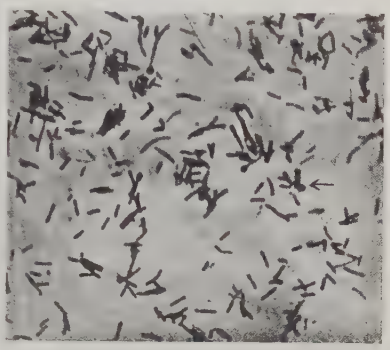

Fig. 5. Five days old culture of strain 2 showing vegetative cells, immature spores (stained) and one unstained mature spore (arrow). Erythrosine. × 800.

The bacteria are usually nonmotile, but occasionally a very few definitely though feebly motile individuals have been observed. The motile cells display an end over end spinning motion rather than a translocation. The type of flagellation has not been determined.

Spore-like bodies are formed by *Mb. Omelianskii*. These bodies are spherical or nearly so and are located terminally where they cause a definite swelling of the rod (Fig. 5). When immature, they stain with ordinary dyes; when mature they are colored only by special spore-staining procedures. For example, when stained by the CONKLIN modification of the WIRTZ method (5) the spore-like bodies, both free and in the cells, are green while the vegetative rods are red. The spore-like bodies display the usual high refractive index characteristic of spores. It seems reasonable to conclude that they are indeed true spores.

Though the spore-like bodies are morphologically like true spores, their heat resistance is unusually low. With pure cultures it has never been possible to obtain growth from inocula heated at 80°C. for 10 minutes, though enrichment cultures have occasionally developed after inoculation with pasteurized mud. In view of the low heat resistance it seems probable that the spores of *Mb. Omelianskii* have some other function than to protect the organism against high temperatures. A much more useful function for the spores of an obligate anaerobic organism would be to allow survival in the presence of oxygen. Probably

the spores of *Mb. Omelianskii* act in this way, for it has been observed that cultures sometimes remain viable after being exposed to air for a month or more, in spite of the fact that vegetative cells appear to be very sensitive to oxygen. However, there is as yet no direct proof that the survival in air is due to spores rather than vegetative cells.

The cells of *Mb. Omelianskii* are variable with respect to the Gram stain. The majority of the cells are completely Gram negative, while 5—20% are distinctly, though weakly, Gram positive, and intermediate degrees of staining can also be found. In fact on several occasions two cells joined together were observed, one of which was Gram positive, the other negative. There is no apparent relation between age or colony type and the Gram stain. Gram positive and negative cells occur in about the same ratio in both the large soft and the diffuse colonies. This is another indication that these two colony types are formed by the same organism.

With erythrosine also, not all cells stain the same. Some are colored lightly and rather evenly; others are colored more intensely and have a granular appearance. There are also all intermediate types of staining. It was at first thought that the cells staining intensely with erythrosine might be the same as those that are Gram positive. This appears, however, not to be correct, for the percentage of Gram positive cells is much smaller than the percentage of cells staining intensely with erythrosine.

3. CONDITIONS INFLUENCING GROWTH.

When pure cultures were first isolated they were transferred to tubes of medium of the following composition (medium A): C_2H_5OH 1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.05%, agar 0.3%, $Na_2S \cdot 9H_2O$ 0.03% and Na_2CO_3 0.35%, made up in tapwater. The sulfide and carbonate were added as sterile solutions and the reaction was then finally adjusted with sterile dilute HCl to pH 6.6—6.8 with the aid of indicators. After inoculation, 0.35 cc of saturated pyrogallol solution and 0.25 cc of 10% K_2CO_3 were added to an absorbent cotton plug and the tubes were stoppered. The temperature of incubation was 35—38°C.

Growth in the above medium was extremely erratic. Sometimes transfers developed rapidly and abundantly; at other times they failed to develop or growth was slow and poor. Since this erratic behavior indicated that the medium was not entirely suitable it was decided to investigate systematically the effect of varying its composition.

Effect of H_2S concentration. Hydrogen sulfide may be expected to exert its effect in two distinct ways. By reacting with

oxygen and reducing the oxidation-reduction potential it should (in accordance with previous observations) make the medium more favorable for growth. On the other hand, H_2S, particularly the undissociated molecule, may be toxic to living organisms. So high concentrations are likely to cause an inhibition of growth. Since the ratio of undissociated H_2S to HS' and S''ions is determined by the pH, the latter factor should influence the toxicity.

These predictable relations were actually observed. In the absence of H_2S growth was greatly delayed or absent. In several experiments no growth occurred; in one, bacteria began to develop only after 25 days' incubation. In this instance, amounts of H_2S or other reducing substances sufficient to allow the initiation of growth may have been introduced with the inoculum. The intentional addition of a small quantity of H_2S (0.8 mg per 100 cc) always permitted rapid and abundant development which was not improved perceptibly by further additions up to 2—3 mg H_2S per 100 cc. High concentrations caused a definite inhibition of growth in a poorly buffered medium (0.1% K_2HPO_4) of initial pH 6.8. The inhibition was almost complete with 8—9 mg H_2S per 100 cc. It may be mentioned that this H_2S toxicity may influence the competition between methane-producing and sulfate-reducing bacteria in a neutral environment in such a way as to favor the latter.

In a more highly buffered medium (1.0—1.5% K_2HPO_4) of higher initial pH (7.6—7.8), the favorable influence of low H_2S concentrations was also observed (Table 1). But at higher H_2S concentrations, up to

Table 1.

Effect of H_2S concentration on growth and acid production.

(K_2HPO_4 1%, initial pH 7.8, incubation period 6 days).

H_2S added mg/100 cc	HAc formed cc 0.1 n/10 cc
0.00	0.00
0.54	0.80
0.97	3.50
1.96	3.58
7.85	3.66
11.8	3.62

11.8 mg per 100 cc, no inhibition was apparent. This lack of toxicity is probably a result of the lower concentration of undissociated H_2S at the higher pH.

From these experiments it can be concluded that a suitable H_2S concentration will be obtained by the addition of 1 cc of a 1% $Na_2S \cdot 9H_2O$ solution per 100 cc of medium. This is equivalent to about 1.4 mg H_2S per 100 cc. The quantity of sulfide originally added to medium A (0.03%) is definitely too high.

Effect of pH. Several preliminary experiments on the effect of pH upon growth were carried out using medium A, modified to contain 0.01% instead of 0.03% $Na_2S \cdot 9H_2O$. The reaction of the medium was varied by suitable additions of a sterile HCl solution. These experiments indicated that the reaction is a very important factor influencing the growth of *Mb. Omelianskii* and that the poor and erratic growth obtained in the earlier cultures was undoubtedly due in large part to an unfavorable reaction. The range pH over which good growth occurred was exceedingly limited, being from about pH 6.7 to 8.2. Since the pH determinations were carried out by the indicator method they are subject to considerable error, probably at least $\pm$ 0.2 units.

A second series of experiments on the relation of pH to growth was carried out using a more highly buffered medium (1.5% instead of 0.1% K_2HPO_4). pH determinations were made with a glass electrode and are accurate to about $\pm$ 0.02 pH unit.

The essential data from two independent experiments are given in Table 2. Experiment 1 covers the pH range near the acid limit for growth. It will be seen that the time required for active gas production to begin is greatly influenced by the initial pH of the medium. At pH 6.54, 4 days were required to initiate gas production. With initial pH 6.75 this was reduced to 2 days and with initial pH 7.12 to 24 hours. All cultures were of course inoculated in the same manner. Other experiments have shown that pH 6.5 is very close to the limiting acidity at which growth can be initiated.

As a result of the metabolic activity of the organism the medium becomes more acid. The decrease in pH in this experiment was quite small, only a few tenths of a pH unit. In less strongly buffered media the pH frequently drops to 5 before acid production stops. But there is no evidence that growth occurs below pH 6.3—6.5.

It seems a little strange that an organism forming an organic acid as one of its principle metabolic products should be so sensitive to acidity.

Generally acid-producing organisms, like the acetic acid and the lactic acid bacteria, are particularly acid tolerant. The explanation for the lack of acid tolerance of this particular organism can perhaps be found

Table 2.

Relation of pH to growth.

(Medium A without agar and with 1.5% K_2HPO_4 and 0.01% $Na_2S \cdot 9H_2O$).

Experiment	Initial pH	Time in Days [1])	Final pH
1	6.54	4	6.15
	6.75	2	6.33
	6.91	1.5	6.55
	7.12	1	6.73
	7.43	2	6.95
2	7.63	1	—
	7.76	2	6.90
	7.84	2	6.95
	7.96	5	6.93
	8.10	11	—

[1]) Period of incubation at 37°C. before first gas production became visible..

in the circumstance that in nature there is an intimate association between acetic acid-forming and acetic acid-fermenting methane bacteria such that acid never accumulates. It is well known that acetic acid is one of the most rapidly fermented organic compounds.

The second experiment of Table 2 covers the pH range in the vicinity of the alkaline limit for growth. It must be pointed out that the absolute time periods required for visible gas production to begin are only comparable within a single experiment. Because of differences in the age and vigor of the inocula the time periods in experiments 1 and 2 cannot be directly compared.

The data of experiment 2 show that the rate of development of the bacteria is greatly decreased as the alkalinity is increased from pH 7.63 to pH 8.10. Only 1 day is required for visible gas production to begin at pH 7.63, while 11 days is required at pH 8.10. The latter reaction is evidently close to the upper limit of tolerance. In other experiments, cultures failed to grow even after a much longer period of time at ap-

proximately the same pH. In order to insure rapid development the initial pH should not be above pH 7.8.

To summarize, good growth of our strain of *Mb. Omelianskii* occurs only over the range of pH 6.7—7.8, the extreme limits being about pH 6.5—8.1. The optimum reaction seems to be pH 7.0—7.2.

As a result of these findings, all media (unless otherwise stated) were subsequently adjusted to an initial pH of 7.6—7.7, *i.e.*, fairly close to the upper limit of pH tolerance. This allows growth to start fairly rapidly and at the same time permits the bacteria to form a relatively large quantity of acetic acid before the pH becomes so low as to be inhibitory. In this way maximum growth is obtained.

Effect of Phosphate Concentration. Since the range of pH suitable for the growth of *Mb. Omelianskii* is so small, it is essential to use a highly buffered medium if one wishes to obtain heavy growth and the conversion of considerable quantities of CO_2 and C_2H_5OH. The upper limit of phosphate tolerance was therefore investigated.

Medium A contains only 0.1% K_2HPO_4. Preliminary experiments showed that much higher concentrations can be tolerated and, in fact, are highly beneficial. Table 3 shows that K_2HPO_4 does not become inhibitory until the concentration exceeds about 2 grams per 100 cc. Consequently, rather highly buffered media can be safely used. In most subsequent experiments a phosphate concentration of 1.0—1.5% was used.

Table 3.

Effect of phosphate concentration.

(Modified Medium A: $Na_2S \cdot 9H_2O$ 0.01%, pH 7.7, Incubation period 7 days).

K_2HPO_4 conc. g/100 cc	Final pH	HAc cc 0.1 n/10 cc
0.91	6.7	4.70
1.14	6.8	4.70
1.36	6.8	4.76
1.82	6.9	4.81
2.27	7.6	0.51

High buffering can, of course, also be obtained by adding an excess

of $CaCO_3$ to the medium. This is frequently undesirable because of the increased difficulty of obtaining samples for analytical work or of separating the bacteria from the carbonate for cell suspension experiments. However, if one wishes simply to obtain a maximum conversion of ethanol to acetic acid, $CaCO_3$ is very suitable since it provides both buffering and carbon dioxide.

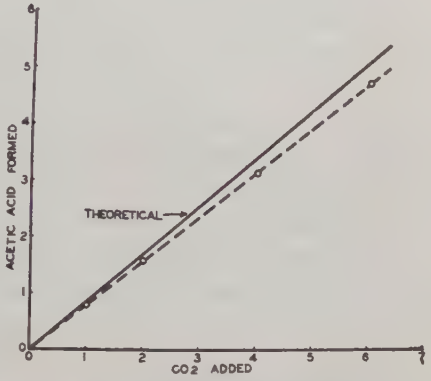

Fig. 6. Relation between available carbon dioxide and acetic acid formation. The ordinate represents the cc of 0.1 n acetic acid formed per 10 cc; the obscissa gives the cc of 5% Na_2CO_3 solution added per 100 cc of medium.

Effect of Carbonate Concentration. Since CO_2 is an essential nutrient for *Mb. Omelianskii*, it is to be expected that increasing the quantity of available CO_2 will correspondingly increase growth and acetic acid formation from ethanol, other conditions being favorable. This found to be true. The data of Table 4 which are plotted in Figure 6, show that acetic acid formation is a linear function of the amount of added sodium carbonate over the concentration range of 0—278 mg Na_2CO_3 per 100 cc. The theoretical values for acetic acid (column 3) are calculated from the equation 2.

$$2\,C_2H_5\,OH + CO_2 \rightarrow 2\,CH_3COOH + CH_4 \qquad (2)$$

It will be seen that the observed acid production is somewhat less

Table 4.

Relation of acid production to carbonate supply.

Na_2CO_3 mg/100 cc	HAc (observed) cc 0.1 n/10 cc	HAc (theoretical) cc 0.1 n/10 cc
0.0	0.00	0.00
46.4	0.79	0.87
92.8	1.60	1.74
185.6	3.16	3.49
278.4	4.78	5.22

than the theoretical. The discrepancy is undoubtedly largely due to the conversion of part (about 5%) of the ethanol into cell material. It will be shown in a separate communication, that the quantity of cell material formed is also directly proportional to the quantity of available CO_2 and is of the indicated order of magnitude.

Although growth and acid production are directly proportional to the carbonate supply over the range considered in the above experiment, it cannot be expected that this relation will also hold for higher carbonate concentrations. As the concentration of carbonate is increased the quantity of acetic acid formed will tend to become so great that the pH can no longer be maintained within a range favorable to growth under the experimental conditions chosen. Acidity will therefore eventually limit alcohol oxidation. At high carbonate concentrations growth may also be inhibited by osmotic or specific ionic toxicities.

Table 5 shows that growth and acid production are inhibited when the Na_2CO_3 concentration in unmodified medium A exceeds 400 mg per 100 cc. With 600 mg per 100 cc growth is almost completely prevented.

Table 5.

Relation of growth and acid production to carbonate concentration.

(Medium A with 0.01% $Na_2S \cdot 9H_2O$. Initial pH 6.8).

Added Na_2CO_3 mg/100 cc	5 days [1])	13 days [1])	
	growth	growth	HAc cc 0.1 n/100 cc
280	+	+ + +	2.68
390	—	+ + +	2.54
460	—	+ +	1.10
600	—	+	0.72

[1]) Period of incubation.

Now this toxicity of Na_2CO_3 can hardly be due to an excessively high osmotic pressure because the concentration is only 5.7×10^{-2} Molal whereas K_2HPO_4 does not become toxic until the concentration is about twice as high, 11.0×10^{-2} Molal. The inhibition of growth is therefore probably due to the toxicity of the sodium or bicarbonate ions or to a lack of balance between these and other ions in the medium.

That the effect is determined at least in part by the lack of balance between sodium and potassium ions is indicated by the data c Table 6. In this experiment modified medium A was used, containing 1% instead of 0.1% K_2HPO_4. The influence of increasing concentrations of Na_2CO_3 and K_2CO_3 was compared.

Table 6.

Relative effects of Na_2CO_3 and K_2CO_3 on growth and acid production.

(Modified medium A: K_2HPO_4 1%, $Na_2S.9H_2O$ 0.01%, pH 7.8).

Salt	mg/100 cc	Molality × 10^2	Growth[1])	HAc[1]) cc 0.1 n/10 cc
Na_2CO_3	276	2.60	+ + +	5.87
Na_2CO_3	387	3.65	+ + +	7.78
Na_2CO_3	497	4.69	+ + +	(5.26)
Na_2CO_3	608	5.73	+ + +	8.16
K_2CO_3	326	2.36	+ + +	5.25
K_2CO_3	456	3.30	+ + +	5.06
K_2CO_3	586	4.24	+	1.41
K_2CO_3	717	5.19	+	1.06

[1]) After 6 days' incubation.

It can be seen that in the presence of 1% K_2HPO_4, 5.73×10^{-2} Molal Na_2CO_3 does not show any toxicity. K_2CO_3, however, inhibits growth at an appreciably lower concentration. These results and those of the previous experiment (Table 5) may be explained as follows: The toxicity of Na_2CO_3 and K_2CO_3 in the concentration range studied is due to the cation. When either the Na or K ion concentration is high, the concentration of the other being low, toxicity results. By maintaining a balance between the two cations higher concentrations of either can be tolerated.

The upper limit of carbonate concentration tolerated by *Mb. Omelianskii* under the most favorable conditions has not been determined. Probably it would not exceed 0.1 M.

Ethanol Concentration. Growth and acid production are not appreciably effected by variations of ethanol concentration in the range 0.4—2.0 vol. %. The upper limit of alcohol tolerance has not

been determined. For most experiments and routine cultures 0.4—1.0 vol. % has been used. Of all the organic compounds utilized by *Mb. Omelianskii*, ethanol appears to be the most favorable.

Nitrogen Source and Growth Factors. Organic nitrogen compounds are not necessary for the growth of *Mb. Omelianskii*. Hundreds of transfers have been made in media containing $(NH_4)_2SO_4$ as the only added nitrogen source and ethanol as the only organic compound. Traces of organic nitrogen compounds may, it is true, have been present in the tapwater generally used in the preparation of media. Special experiments, however, have shown that growth also occurs when tapwater is replaced by double distilled water. It must be concluded that this organism is able to synthesize all of its nitrogenous cell constituents from ammonia nitrogen.

Variations in $(NH_4)_2SO_4$ concentration from 0.02—0.2% were found to have little or no influence upon growth. The lowest concentration was, if anything, the best. 0.03% $(NH_4)_2SO_4$ supplies a quantity of nitrogen well in excess of the quantity required for maximal growth in the media used. This concentration was generally chosen. It may be mentioned that nitrate, except in very low concentrations, is toxic.

Although organic nitrogen compounds are not essential, it was thought possible that complexed nitrogenous extracts like yeast extract might improve growth by providing a more readily available nitrogen source or by supplying growth factors not synthesized in optimal amounts. A number of experiments were therefore undertaken with media to which various amounts of yeast extract were added. In no instance was any favorable effect of the extract observed with respect to either the rate or amount of growth. Low concentrations of yeast extract, up to 0.1% on a dry weight basis, neither improved or retarded growth. Higher concentrations up to 0.9%, were somewhat inhibitory. With 2.7% yeast extract no growth occurred.

The above results appear to be somewhat at variance with the observation of Heukelekian and Heinemann (4), that a small amount of yeast extract considerably decreases the time required for gas production in media used for counting methane-producing bacteria in sewage sludge. However, it must be remembered that their experiments are not strictly comparable with ours. They worked only with crude cultures in which the methane-producing ethanol-fermenting bacteria may have been quite different from our strains. Also the presence of

other types of bacteria introduces a possible complication in the interpretation of their results.

A g a r. Semisolid agar (0.1—0.3%) has been used in preference to liquid media in many experiments. By eliminating convection currents, and possibly in other ways, agar appears to facilitate growth, particularly under conditions where the medium is not completely protected from access of oxygen. Thus, it has proved possible to grow strain 2 in semisolid media in open tubes unprotected from the air, provided the tubes were inoculated fairly heavily immediately after being autoclaved. The bacteria generally fail to develop in liquid media exposed to air. However, when oxygen is carefully excluded, liquid cultures develop as rapidly and as abundantly as those with agar. They have been extensively used for the preparation of cell suspensions for various physiological experiments. Both pyrogallol made alkaline with K_2CO_3 and Oxsorbent (an acid chromous chloride solution) have been used successfully as oxygen absorbents. A paraffin seal may also be used.

In connection with the use of liquid media, reference should perhaps be made to the idea that large quantities of inert sediments are essential for the growth of methane bacteria. With our organisms no such requirement exists. Excellent growth invariably occurs in media to which no special sediment (such as asbestos or calcium carbonate) has been added and which contain only small precipitates of calcium phosphate and iron sulfide. In such media the bacteria develop not only in the sediment but throughout the liquid, producing a general turbidity. Undoubtedly sediments could be eliminated entirely without any deleterious effects provided care was taken to supply an adequate amount of iron, calcium and other essential elements.

T e m p e r a t u r e. Cultures were incubated at various temperatures at 3°C. intervals from 16°—45°C. In a 30-day incubation period no growth occurred at temperatures below 26°C. At this temperature growth was very slight. Highest growth rates were observed at 37° and 40°C.; the optimum is probably nearer the latter temperature. At 45° development was slower, about the same as at 31°C. The maximum temperature for this culture is probably near 48°C.

It should be pointed out that these temperature characteristics were determined after the organism had been cultivated for more than a year at 35—37°C. Consequently it is quite possible, if not probable, that the

original temperature characteristics of the organism had become modified by adaptation to higher temperatures. Under natural conditions the temperature to which this organism would be exposed could hardly exceed 25° C., which is below the determined minimum. In this connection it is interesting to note that HEUKELEKIAN and HEINEMANN found ethanol-fermenting bacteria obtained directly from sewage sludge to have a temperature optimum of 30—35°C.

4. A CULTURE MEDIUM FOR *Mb. OMELIANSKII.*

On the basis of the above experiments the following medium has been developed and found to be very satisfactory for the cultivation of *Mb. Omelianskii.*

Medium B. C_2H_5OH 0.4—1.0 vol. %, K_2HPO_4 0.6%, KH_2PO_4 0.9%, $(NH_4)_2SO_4$ 0.03%, $MgSO_4 \cdot 7H_2O$ 0.01%, $FeSO_4 \cdot 7H_2O$ 0.001% and $CaSO_4$ 1% of a saturated solution. Distilled water may be used, though tapwater in general gives better results; with the latter both the $CaSO_4$ and the iron may be unnecessary. Agar may be added as desired. Immediately after autoclaving add per 100 cc 1—2 cc of 1% $Na_2S \cdot 9H_2O$ solution and 5—8 cc of 5% Na_2CO_3 solution. This should give a reaction of pH 7.4—7.7. The sulfide and carbonate solutions should be autoclaved along with the medium to free them of dissolved oxygen; it is sometimes convenient to mix the sulfide with the carbonate before autoclaving. In any event the sulfide solution should not be more than a few days old as it slowly oxidizes. Inoculate the medium immediately with 0.1—1.0 cc (more or less may of course be used) of a young culture (preferably not more than one week old). Apply a pyrogallol — K_2CO_3 or Oxsorbent seal and incubate at 35—40°C.

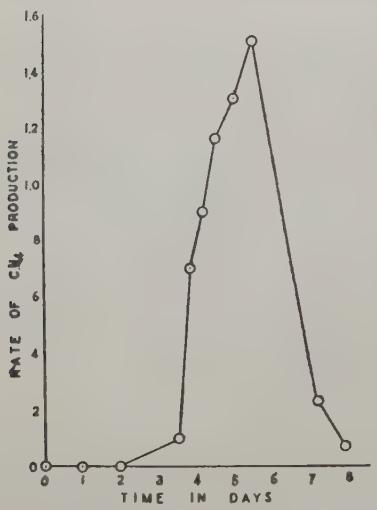

Fig. 7. Rate of methane production by a liquid culture of *Mb. Omelianskii.* 37° C. The ordinate units are cc of methane per 100 cc of medium per hour.

The above medium may be modified in various ways. If it is desired to obtain a large cell yield the buffering capacity should be increased as much as possible by adding more phosphate (up to 2%) and adjusting

the reaction with carbonate to pH 7.8. To obtain most rapid growth it is better to adjust the reaction to pH 7.0—7.2. The quantity of sulfide depends upon whether or not agar is used. With semisolid or solid agar 1 cc of 1% sulfide solution is entirely satisfactory; with liquid cultures, particularly those of small volume, 1.5—2.0 cc may be preferable.

In order to illustrate the behavior of *Mb. Omelianskii* in medium B, typical data on the gas evolution from a liquid culture are given in Fig. 7. The rate of methane evolution is plotted as a function of the time of incubation at 37°C. In this culture gas production began after about 2 days and reached a maximum in about 5 days. The fermentation was complete after 8 days. Analysis of the medium (500 cc) at this time showed that all of the CO_2 (about 850 mg) had disappeared and a little over 2 g of acetic and 145 mg dry weight of cells had been formed. In some fermentations visible gas production begins within 24 hours after inoculation and is completed in 3—4 days. As has already been indicated the speed of fermentation depends primarily upon the pH of the medium and the vigor of the inoculum.

Summary.

1. The isolation of pure cultures of *Mb. Omelianskii* has been achieved by the agar shake culture method. The purity of the cultures was established by microscopic examination and by testing for possible contaminants in suitable media under both aerobic and anaerobic conditions.

2. The organism is a thin, unbranched, Gram-variable rod. Motility occurs but is rare. Spores of low heat resistance are formed.

3. Growth of the organism in pure culture is shown to be influenced by a number of factors including hydrogen sulfide concentration, pH, phosphate and carbonate concentrations. The pH range of growth is from 6.5 to 8.1, the optimum being at 7.0—7.2. Increasing the phosphate concentration up to about 2% is beneficial because it increases the buffering of the medium. Growth and alcohol oxidation are directly proportional to the carbonate supply at low concentrations. High concentrations of soluble carbonates are inhibitory.

4. *Mb. Omelianskii* uses ammonia as a nitrogen source and is not stimulated by organic nitrogen compounds or growth factors such as are present in yeast extract.

5. A culture medium is described which allows rapid and abundant development of *Mb. Omelianskii* quite comparable with that obtainable with other obligately anaerobic bacteria.

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(From the Department of Internal diseases of the University Hospital, Groningen).

THE DEVELOPMENT OF SULFANILAMIDOPYRIDINE-RESISTANT STRAINS OF PNEUMOCOCCI IN VIVO

by

J. MULDER

(Received March 21, 1940).

Already in 1938 WHITBY (6) assumed on clinical grounds that sulfanilamidopyridine-sensitive strains of micro-organisms could become resistant against this drug in vivo.

MACLEAN, ROGERS and FLEMING (3) subsequently demonstrated that a pneumococcus strain type 8, in itself fairly resistant against sulfanilamidopyridine, rapidly acquired a high degree of resistance by way of mouse-passage in animals treated with this drug.

ROSS (5) found that a pneumococcus strain type I, isolated by BACKHOUSE during the first 24 hours of a fatal meningitis from a native child in New Guinea, was sulfanilamidopyridine-sensitive; but the same strain isolated from the child after three days of intensive treatment with sulfanilamidopyridine proved resistant against the drug.

MAC LEOD (4) described the development of drug-resistant strains in mice.

In the past months we were able to demonstrate how a highly sulfanilamidopyridine-sensitive pneumococcus strain (type I) rapidly became resistant against the drug in the mouse, and how it could be raised to an absolute resistance without impairing the virulence of the strain.

TECHNIQUE.

The pneumococcus strain used by us, was a type I strain, obtained from the Neufeld Laboratory of the Robert Koch Institute of Berlin, where it is named America Type I. This strain was grown by NEUFELD and HÄNDEL in 1909, and later sent to the Rockefeller Institution.

The therapeutic effect of the oral administration of sulfanilamidopyridine in infected mice was traced by daily inoculating a loopful of blood from the tail on blood-plates, and counting the resulting pneumococcus colonies. In this way, day by day, the approximate degree of infection of the mouse was determined.

The mice were injected intraperitoneally with dilutions of a highly virulent 18-hours-old culture in serum broth, 1 ml of which contained 10^8—10^9 organisms. The doses of sulfanilamidopyridine mentioned in the figures, are those for mice of 20 grams.

SENSITIVITY OF THE PNEUMOCOCCI TO SULFANILAMIDOPYRIDINE.

The type I strain was found to be very sensitive to sulfanilamidopyridine (see Table I and fig. 1).

Table I.

Sensitivity of the strain America I to sulfanilamidopyridine.

Minimum observation time of surviving mice 12 days. The drug was administered once daily (40 mg).

Experiment	Inoculum (number of pneumococci)	Number of mice	Died	Survived	M.L.D. (number of pneumococci)
I.	$1{,}3 \times 10^7$	1	1	0	
	$1{,}3 \times 10^6$	3	0	3	
	$1{,}3 \times 10^5$	2	0	2	6
	$1{,}3 \times 10^4$	2	0	2	
II.	6×10^6	4	3	1	
	6×10^5	6	3	3	6

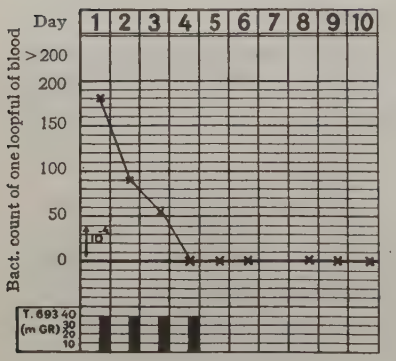

FIG. 1. Course of the bacteraemia of a mouse after treatment with sulfanilamidopyridine 6 hours after intra-abdominal infection with the strain America I. The number on the left indicates the number of colonies growing from one loopful blood from the tail.

DEVELOPMENT OF SULFANILAMIDOPYRIDINE-RESISTANT STRAINS IN THE MOUSE.

In one of several mice treated orally with 40 mg of the drug six hours after the infection (10^{-4}), and on the four subsequent days, the bacteraemia did not entirely disappear, and the mouse died on the eighth day of the experiment (see fig. 2*A*).

On the sixth day, the pneumococcus strain grown from the blood

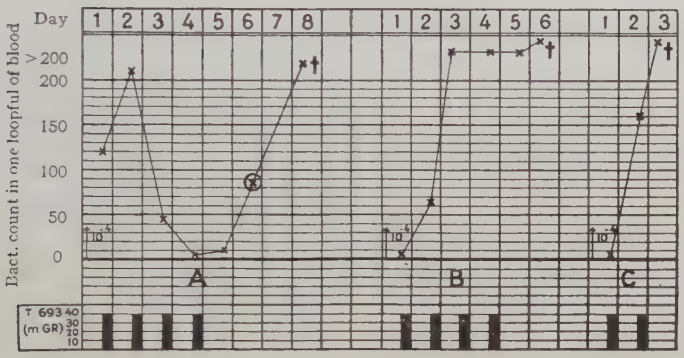

FIG. 2. Development of a sulfanilamidopyridine-resistant strain (America type I) in mouse A. In mouse B and C the strain is a drug-resistant one. The sulfanilamidopyridine was administered 6 hours after infection.

of the mouse's tail was injected in a 10^{-4} dilution into two other mice. These mice were treated with sulfanilamidopyridine six hours after the infection, and subsequently daily for 2 and 4 days respectively. Though six hours after the infection the bacteraemia in both mice was very low (under five colonies per loopful of blood, which might be explained by a reduced virulence), a serious bacteraemia developed afterwards in both animals, and they died after 2 and 5 days respectively (fig. 2*B* and *C*). From this it may be concluded that the strain had already become resistant in the first mouse within the 5 days during which the drug was administered. In fig. 3 an other experiment is recorded.

The strain was now passed through mice with intercalated subculturing in serum broth, to which at first 4 mg pct of sulfanilamidopyridine was added, which was afterwards omitted, as it proved to be unnecessary for maintaining the resistance. A daily dose of 40 mg was administered orally to the mice until they died.

After a series of passages with three interruptions, during which the resistant strain was preserved in a dried spleen, an absolute

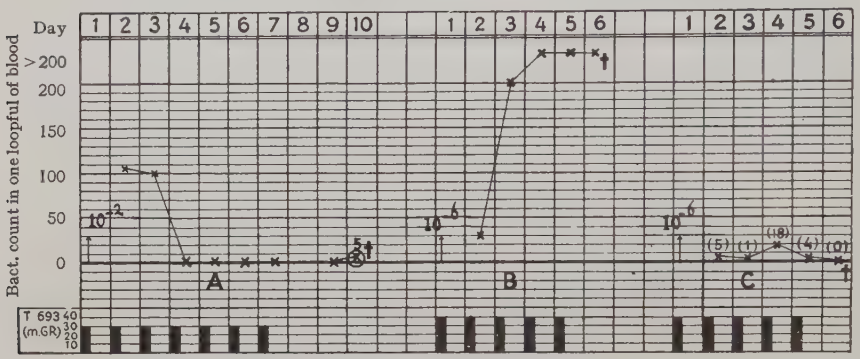

FIG. 3. Development of a second drugresistant strain (America I) in mouse A. The drug was administered at the time of infection. The strain from mouse A turns out in mouse B to be a completely resistant one; in mouse C it is a partially resistant one.

resistance was ascertained, the virulence of the strain being fully maintained (Table II).

Table II.

Complete resistance to sulfanilamidopyridine of the fast-strain America I.

The mice were treated once daily with 40 mg of the drug.

Inoculum Average number of pneumococci in 3 experiments	Number of mice	Died	Survived
6	12	12	0

The course of the infection with the young sulfanilamidopyridine-resistant strain in mice was very protracted in the first passage. A remarkable feature of this protracted development was the presence of bacteraemia, so that the mice showed highly positive blood-cultures, often over 200 colonies per loopful, for several days until they died. Fig. 4 shows a survival-time of 12 days of an infected mouse (8th passage; inoculation dose 10^{-3}).

The phenomenon of long survival, notwithstanding the presence of

bacteraemia, became less marked after further passages probably in connection with the virulence.

Possibly in vitro experiments on the method described by Wolff and Julius (7), carried out parallel with the above in vivo experiments, can throw some light on the matter. It will be of future interest to investigate how long the property of resistance is maintained. After five passages through mice we found that the resistance had lost none of its power.

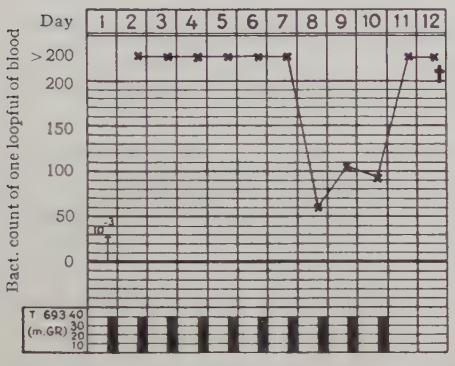

Fig. 4. Course of the bacteraemia after inoculation of a large dose of the young resistant strain (8th passage).

In our opinion the clinical consequences of experiments of this nature are of great importance; and we will have to count with the fact that the resistance of a pneumococcus strain can develop very rapidly in the body. Serious cases of pneumococcal infection (sepsis, meningitis, peritonitis), must therefore immediately be treated with very large doses of sulfanilamidopyridine, may be better still, in combination with type-specific serum. When the course of the infection becomes subacute, as a.o. is sometimes the case with meningitis, there is a possibility of the strain having become resistant (cf. Ross (5)).

If in such a case an anti-serum has not yet been given, it may be useful to do so at a later stage (Cutts, Gregory and West (1), Dicke (2)).

Summary.

1) In mice the pneumococcus strain America type I may become resistant against sufanilamidopyridine within a few days, in spite of the fact that this strain originally is highly sensitive to this drug.

2) The young resistant strain is at first less virulent than the sensitive strain, but after several mouse-passages it can easily regain its full virulence.

3) It is possible to make the strain wholly resistant against sulfanilamidopyridine.

4) The young resistant strain may cause a protracted bacteraemia

in mice when the latter are treated with the drug. Later on this phenomenon for the greater part disappears.

5) In clinical practice it is of great importance to count with the development of drug-resistance, and it will be wise to treat serious pneumococcal infections immediately with substantial doses of sulfanilamidopyridine, probably better still in combination with an anti-serum.

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(From the Laboratory of Microbiology, Technical University, Delft).

ON THE INCIDENTAL OCCURRENCE OF ROD-SHAPED, DEXTRAN PRODUCING BACTERIA IN A BEET-SUGAR FACTORY

by

L. H. C. PERQUIN

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1. INTRODUCTION.

During the last beet-sugar campaign the ,,Cooperatieve Beetwortelsuikerfabriek Roosendaal'' at Roosendaal (Holland) approached Prof. KLUYVER with the request to examine the nature of gelatinous grains which had spontaneously occurred in the raw beet juice. This mass had given rise to serious interruptions in the manufacturing process by clogging pipe-lines through which the juice passed.

In consequence of this request various investigations have been made by the present author, the results of which are reported below.

A superficial examination of the forwarded sample of grain-conglomerates made it extremely acceptable that we were dealing here with a case of so-called ,,Froschlaich'' (,,frogs' spawn'') as frequently reported in literature. As is well known, the formations which are indicated by this name are due to the development in the beet juice of a coccus-shaped lactic acid bacterium usually designated as *Leuconostoc mesenterioides* (Cienkowski) van Tieghem, and in more recent papers as *Betacoccus arabinosaceus* Orla-Jensen. This organism is characterized by the fact that when grown on saccharose-containing media the cells are surrounded by a thick cell wall or capsule. Several of these capsules are usually united to conglomerates [1]), thus forming smaller or larger solid grains.

It was, therefore, more or less a surprise to us that a microscopic examination of the ,,Froschlaich'' in question revealed that the grains were built-up by distinctly rod-shaped bacteria surrounded

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[1]) Fine photographs of such conglomerates are to be found in a publication of ZETTNOW (32).

by thick capsules. This is clearly seen in Fig. 1 which gives a photographic reproduction of a nigrosine preparation of a crushed grain. The rod-shape of the bacteria is still more easily distinguishable in Fig. 2 which brings a preparation after it had been stained according to Gram.

Fig. 1. Encapsulated rod-shaped cells in a nigrosine preparation of a crushed grain. × 1050.

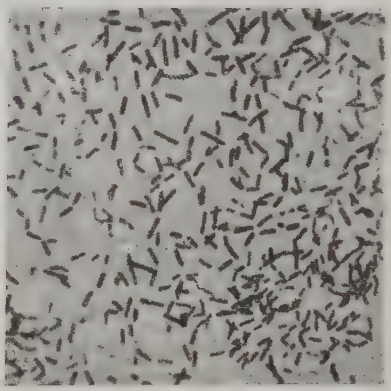


Fig. 2. Rod-shaped cells in a crushed grain, stained according to Gram. × 1050.

This unexpected result made it desirable to make a thorough study of previous publications in which rod-shaped bacteria have been described as being responsible for mucus production in saccharose-containing juices. A survey of this literature is given in Chapter 2.

2. LITERATURE ON THE FORMATION OF MUCUS IN SACCHAROSE-CONTAINING MEDIA BY ROD-SHAPED BACTERIA.

First of all it should be stressed that this survey only will deal with rod-shaped bacteria for which mucus production is confined to the presence of saccharose in the medium. In other words, all publications dealing with bacteria capable of rendering most unspecific media slimy, will be left out of consideration here.

Besides giving particulars regarding the various bacteria for which the properties of forming mucus out of saccharose has been claimed, also attention will be given to what has been reported regarding the chemical nature of the mucus.

In this connection it should first be remarked that already in 1874 SCHEIBLER (25) had given experimental proof that the chemical con-

stitution of the common „Froschlaich" as occurring in sugar-factories was that of a glucose polymer, for which he introduced the name of „d e x t r a n". Since it has been confirmed by several investigators that this conclusion indeed holds for the capsular mass produced by *Leuconostoc mesenterioides* from saccharose. Recently PEAT, STACEY and SCHLÜCHTENER (20), and STACEY and YOUD (26) have brought experimental proof that dextran consists of a linear chain of several hundred glucose units in a gentiobiose linkage.

LIPPMANN (16), however, was the first to observe that not in all cases mucous substances produced by bacterial action out of saccharose had the chemical constitution of dextran. He describes a case in which the mucus formed by a badly defined bacterial species on hydrolysis yielded exclusively laevulose. For the polysaccharides of this type he introduced the name of „l a e v u l a n".

We will now proceed to give a survey of the various rod-shaped bacteria for which the property under discussion has been reported.

As far back as 1889, KRAMER (14) described a facultative anaerobic, non-spore-forming bacterium, to which he gave the name of *Bacillus viscosus sacchari*. This organism was able to transform in one or two days beet juice into a viscous mass by the formation of a slimy substance of the empirical composition $C_6H_{10}O_5$. In a saccharose-containing medium besides the mucus also CO_2 and mannitol were formed. Shortly afterwards RITSERT (23) described a spore-forming, rod-shaped bacterium, *Bacterium gummosum*, that also forms mucus exclusively from saccharose. In 1893 HAPP (9) also isolated a spore-forming, rod-shaped bacterium, *Bacillus gummosus*, that again had the ability to form slime from saccharose. This mucus consisted of a substance soluble in water, insoluble in alcohol and ether, and had the composition $(C_6H_{10}O_5)_n$. Mannitol, lactic acid, butyric acid and CO_2 are reported to have been found as products of the saccharose fermentation.

In 1894 KOCH and HOSAEUS (13) publish a paper entitled: „Ueber einen neuen Froschlaich der Zuckerfabriken", in which they state that in a gelatinous mass, completely analogous with the *Leuconostoc*-„Froschlaich", microscopical examination revealed the presence of a rod-shaped bacterium instead of a coccus-shaped one. The said authors, however, did not succeed in isolating the micro-organism responsible for the „Froschlaich"-formation, but nevertheless gave it a name, *viz.*, *Bacterium pediculatum*. KOCH and HOSAEUS call attention to the fact that the capsules they observed strongly reminded them of the capsules formed by *Bacterium vermiforme*, a bacterium that was iso-

lated two years earlier by WARD (29) from the „ginger-beer plant".

Worth mentioning is that KOCH and HOSAEUS incidentally remark that a chemical analysis should have shown that their „Froschlaich" p a r t l y consisted of laevulan.

In 1895 GLASER (6) described a motile, rod-shaped bacterium — *Bacterium gelatinosum betae* — isolated from a slimy mass, resembling the zoogloea of *Leuconostoc* found in beet juice. Though GLASER does not state explicitly that the said bacterium forms endospores, he mentions that it stands a prolonged heating at a temperature of 100° C. Besides the mucus this bacterium produces from saccharose alcohol and carbon dioxide, but remarkably, no lactic acid was found in the cultures.

In 1897 WARD (30, 31) published an interesting paper in which he describes a remarkable association of organisms, obtained by him in Paris, and said to have come from Madagascar, where it was found as an excrescence on the sugar-cane. „It consists of a bacterium associated with at least one yeast, and grows in saccharose solutions, producing clumps so like the ginger-beer plant that the assumption seemed warranted that we had here a symbiosis of the same kind as that proved to occur there". It is clear that WARD isolated here directly from sugar-cane *Bacterium vermiforme*, the same bacterium he had discovered some years before in the „ginger-beer plant".

POUPÉ's (22) description of a bacterium he isolated from slime in a sugar-factory, is rather vague. The rod-shaped bacterium, which was motile, and easily formed spores, produced a slime that showed much resemblance to that of *Leuconostoc mesenterioides*.

A remarkable bacterium is described by LAXA (15). This facultative anaerobic, thermophilic, spore-forming, rod-shaped bacterium, to which the name of *Clostridium gelatinosum* was given, is according to LAXA of general occurrence in beet juice. LAXA is of the opinion that the zoogloea-formation in beet juice is often wrongly ascribed to *Leuconostoc mesenterioides*, this formation in reality being due to *Clostridium gelatinosum*. The mucus formed by this species yielded on hydrolysis fructose. Lactic acid, volatile acids, and alcohol were found as fermentation products. LAXA considers *Clostridium gelatinosum* as a transition form of a butyric acid bacterium on the one hand, and of *Bacillus subtilis* or *Bacillus mesentericus* on the other hand.

Another extensive investigation was published by GREIG SMITH (8) in 1901, dealing with a *Bacillus* species isolated by him from slimy cane juice. GREIG SMITH states that the species — *Bacillus levaniformans* —

is in all probability a variety of *Bacillus vulgatus*. This bacterium forms high, transparent, gelatinous colonies on agar with 20% saccharose. In a saccharose-containing medium, a one centimeter thick layer of a gelatinous material was formed on the bottom of the flask, the yield of which amounted to about 35% of the saccharose added. Besides this, carbon dioxide and a large quantity of lactic acid, as well as a smaller amount of volatile acid was produced. The polysaccharide that was formed, and called levan by GREIG SMITH, yielded only fructose on hydrolysis. This levan was not formed from glucose, fructose, lactose, or maltose. According to GREIG SMITH, *Bacillus levaniformans* is at the same time a lactic acid and a butyric acid bacterium. In an investigation carried out by STEEL (27) to determine the chemical constitution of levan, it is pointed out that levan, when hydrolyzed, quantitatively yields fructose. STEEL gives, however, some arguments in favour of the view that levan is not identical with the polysaccharide laevulan as earlier described by LIPPMANN (16).

In 1905 MAASSEN (17) published an investigation, in which four species (*S. commune*, *S. citreum*, *S. flavum* and *S. rubrum*) are classified in the new genus *Semiclostridium*. Of the four species especially *S. commune* has a pronounced ability to form gelatinous lumps in saccharose-containing media, which lumps show a great resemblance to the „Froschlaich" formed by *Leuconostoc*. The *Semiclostridium* species are rod-shaped, motile, and form endo-spores. The slime-mass, which on hydrolysis yields fructose, is exclusively formed from saccharose, not from galactose, lactose and raffinose. Besides carbon dioxide, alcohol, lactic acid and volatile acids (no butyric acid) are formed from saccharose and the hexoses.

In 1907 GONNERMANN (7) described two presumably new bacterial species which produce jelly-like masses in beet juice. They are described under the provisional names of *Myxobacillus Betae* and a *Plennobacterium*, and appear to be closely related to *Bacillus subtilis*. An investigation of FERNBACH and SCHOEN (5) of the year 1912, gives likewise incomplete data regarding a facultative anaerobic bacterium — provisionally called gommobacter — that in a suitable saccharose-containing medium converts approximately 50% of this sugar into a gum. On hydrolysis this gum yielded fructose, and accordingly must be considered to be a laevulan. Gum production only took place from saccharose.

In a publication of 1912 BEIJERINCK (2) gives a review of his experiences with dextran-, laevulan-, and cellulan-producing bacteria. Be-

sides his well-known accumulation methods for dextran-forming, coccus-shaped bacteria, he gives a detailed description of a procedure for the isolation of rod-shaped, spore-forming bacteria that form laevulan. He, moreover, stresses that laevulan production is a rather common property of many aerobic spore-forming bacteria. As typical laevulan bacteria BEIJERINCK mentions *Bacillus mesentericus vulgatus*, *Bacillus megatherium* and the new species *Bacillus emulsionis*, which was found to be very common in commercial beet- and cane-sugar. BEIJERINCK too emphasizes that laevulan production only takes place from saccharose and raffinose, not from any other sugar.

In view of BEIJERINCK's investigation it is probable that organisms like *Bacterium gummosum* Ritsert, *Bacillus gummosus* Happ, the bacterium isolated by POUPÉ, *Myxobacillus Betae* and the *Plennobacterium* of GONNERMANN, as well as the gommobacter of FERNBACH and SCHOEN, all are *Bacillus* species with a more or less pronounced laevulan-forming ability. It is difficult to give a definite judgment with respect to *Bacillus viscosus sacchari* Kramer.

It is quite remarkable that, as far as I could ascertain, in the years 1912—1936, no mention is made in literature of interruptions in the sugar manufacture caused by rod-shaped bacteria. In the year 1936, however, SACCHETTI (24) publishes an extensive report on bacteria found by him in the „Froschlaich" in an Italian sugar-factory. He showed that the responsible bacteria could be identified with *Bacillus vulgatus*. The polysaccharide that was formed proved to be laevulan, as only fructose was formed on hydrolysis.

SACCHETTI also arrives at the conclusion that in all probability part of the earlier described bacteria mentioned above, *viz.*, *Bacterium gelatinosum betae* Glaser, *Clostridium gelatinosum* Laxa, *Bacillus levaniformans* Greig Smith and *Semiclostridium commune* Maassen will be identical with or closely related to *Bacillus vulgatus*. Given special conditions these bacteria should be capable of a rapid multiplication in sugar containing juices, forming the characteristic „Froschlaich". The fact that the authors of these bacteria have established that the polysaccharide found in the beet juice proved to be built up from fructose, is in accordance with this conclusion.

SACCHETTI's statement that the *Bacillus* species isolated by him is characterized by acid production (lactic acid and volatile acids) besides alcohol and gas, however, asks for confirmation, since formation of these metabolic products is not known for the more extensively studied strictly aerobic representatives of the genus *Bacillus*. On the other

hand also GLASER, LAXA, GREIG SMITH and MAASSEN report the formation of special metabolic products (lactic acid, volatile acids, alcohol and carbon dioxide).

We may summarize the foregoing survey by concluding that practically all data are in agreement with the view that the well-known „Froschlaich"-formation in sugar-factories may not only be caused by the coccus-shaped *Leuconostoc mesenterioides*, but also by *Bacillus vulgatus* or closely related spore-forming rods. In the first case the polysaccharide produced (dextran) is built up from glucose, in the second case the polysaccharide formed (laevulan) is built up from laevulose.

3. CHEMICAL EXAMINATION OF THE „FROSCHLAICH"-SUBSTANCE.

In view of the considerations in the foregoing chapter it seemed of importance to investigate first the chemical nature of the „Froschlaich"-sample received. Hereto a large part of the available capsular mass was rinsed with tap water during 24 hours in a beaker; the separate grains sank to the bottom of the beaker while the lighter, polluted parts were suspended and carried away by the running water, together with other water-insoluble substances. After 24 hours the grains were separated with a pincette from the coarse sugar-beet cuttings which had also collected on the bottom of the beaker. Part of the grain mass, thus purified, was then dried during 24 hours at a temperature of 45° C., whereupon some grams of the dried mass were thoroughly pulverized in a mortar. Subsequently 520 mg of this powder were hydrolyzed by boiling during 70 minutes in 50 cc 3% HCl under reflux. The slightly coloured solution was then neutralized with 10% KOH and filled up to 100 cc with distilled water. In a polarimeter tube of 189.4 mm a dextrorotation of 0.48° was established. Assuming that the solution only contained glucose, $[\alpha]_D = 52.5°$, it follows that 100 cc of the solution contained 483 mg glucose. In other words, 483 mg glucose had been formed from 520 mg of capsular substance.

This result justifies the conclusion that the „Froschlaich"-substance in question consists of a glucose-polymer which may well be identical with the dextran as produced by *Leuconostoc mesenterioides*.

Taking into consideration the conclusion attained in Chapter 2, the outcome of the chemical examination is quite unexpected, since the presence of rod-shaped bacteria had suggested that we should have been dealing with laevulan.

Under these conditions it became quite interesting to attempt the

isolation and identification of the bacteria responsible for the mucus production.

4. THE ISOLATION OF THE BACTERIA.

After the mass of grains had been thoroughly rinsed with water, as described above, two kinds of grains could be clearly distinguished. Next to the majority of comparatively soft, white to grey grains, there also were a number of smaller, grayish, rock-shaped, hard grains. Microscopic examination showed that both kinds had been built up from encapsulated rod-shaped bacteria. From each type of grain a specimen was thoroughly suspended in sterile water and from these suspensions — resp. from the beet juice in which the sample had arrived — streaks were made on yeast-gelatine with 10% saccharose, on yeast-agar with 10% saccharose, and in some cases on yeast-agar with 2% glucose and 2% chalk. At first peptone-agar was also used. On this medium only very small colonies had developed after a few days. This result at once excluded the possibility that some *Bacillus* species related to *Bacillus vulgatus* was the essential organism responsible for the capsule formation.

The colonies that had appeared on the plates with 10% saccharose and on the yeast-agar-glucose-chalk plates were subjected to a microscopical examination, while special attention was naturally paid to the outward appearance of the colonies on the yeast-gelatine with 10% saccharose. A dextran-forming bacterium, in fact, might be expected to show pronounced slime production on this saccharose-containing plate. Both slime-forming and non-slime-forming colonies appeared to be present.

We cannot dwell here in detail on the results of our examination of the various colonies, but it may be emphasized that by far the majority of the bacteria isolated from the above mentioned soft grains appeared to be gram-positive, catalase-negative rods, besides which, however, there was a small minority of coccus-shaped lactic acid bacteria, that also produced slime from saccharose, and which in all probability could be identified with *Betacoccus arabinosaceus*. The latter species, however, was less rare among the colonies obtained from the hard grains, although here too rod-shaped lactic acid bacteria were predominating.

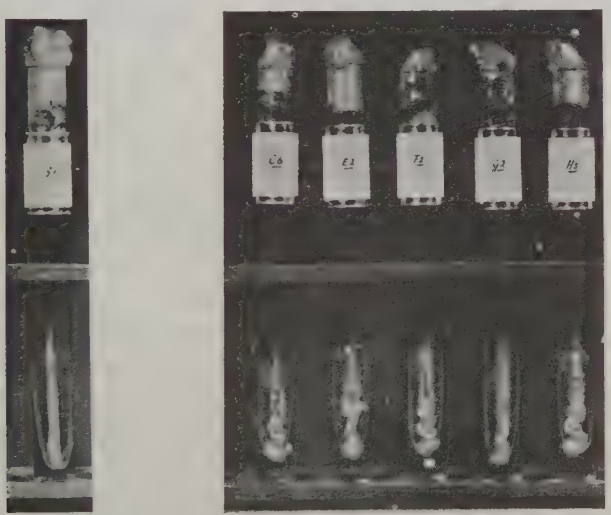
In total 18 strains of rod-shaped bacteria were isolated from a number of grains. All of these were found to be non-spore-forming, gram-positive and catalase-negative which suggested that they all might be rod-shaped lactic acid bacteria. On the basis of a number of characte-

ristics, these 18 strains — A1, B1, B2, C1, C2, C3, C4, C5, C6, E1, E2, F1, F2, G1, G2, H1, H2 and H3 — could provisionally be divided into three groups, a description of which is given in Chapter 5.

5. FURTHER DESCRIPTION AND IDENTIFICATION OF THE ISOLATED STRAINS.

Group I is formed by those strains that distinctly produced acid on yeast-agar-glucose-chalk, but did not form dextran from saccharose. To this group belong the strains: A1, B2, C1, C2, C3, C4, C5, E1 and H1.

The strains under group II also distinctly produced acid on yeast-agar-glucose-chalk, but moreover produced great quantities of dextran both on yeast-gelatine with 10% saccharose (at 20° C.), and on yeast-



Figs. 3 and 5. Respectively strain G1 of group II, and strains C6, E2, F2, G2 and H3 of group III on yeast-gelatine with 10% saccharose.

agar with 10% saccharose (at 30° C.). To this group belong the strains: B1, F1, G1 and H2. A culture of strain G1 on yeast-gelatine with 10% saccharose is reproduced in fig. 3; the slime formed by this strain has the consistency of butter. The slime produced by the other strains of this group has a still somewhat weaker consistency, and ultimately collects on the bottom of the culture-tube, if this is kept in a vertical position. All these strains did not show any capsule formation.

The strains of the remaining group III — C6, E2, F2, G2 and H3 — hardly grew, or even not at all, under aerobic conditions, on yeast-agar

with 2% glucose and 2% chalk, or on yeast-agar with 2% glucose. On the other hand, they developed quite readily on yeast-agar with 10% saccharose. Such a plate culture is reproduced in fig. 4; the colonies resemble hemisperical glass beads. Also the growth on yeast-gelatine with 10% saccharose is most characteristic, and strongly reminds of the behaviour of the capsule-forming strains of *Betacoccus arabinosaceus* on this medium. Cultures of these strains on yeast-gelatine with 10% saccharose are reproduced in fig. 5.

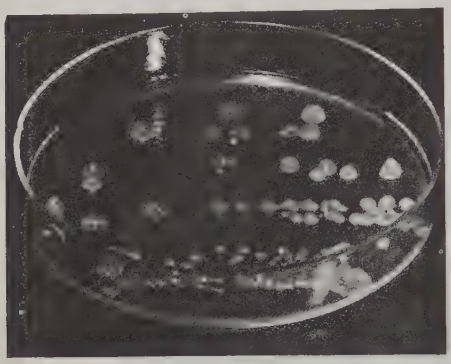


Fig. 4. Plate-culture of strain H3 of group III on yeast-agar with 10% saccharose. × 0.5.

The most remarkable point, however, is that microscopical examination of the colonies either from yeast-gelatine-saccharose or from yeast-agar-saccharose plates revealed the presence of a large number characteristically encapsulated rods which showed a perfect resemblance to those which are microscopically observed in a crushed „Froschlaich"-grain. The resemblance is such that there can be no doubt that the organisms in question are the main constituent of the said grains.

Since all strains appeared to belong to the large group of rod-shaped lactic acid bacteria, they should, according to BERGEY (1), be classified within the genus *Lactobacillus*. However, it is difficult to understand, why BERGEY has not yet accepted a generic sub-division of the rod-shaped lactic acid bacteria as has been done in the excellent monograph by ORLA-JENSEN (19). This scientist has clearly defined three separate genera, to wit *Thermobacterium*, *Streptobacterium* and *Betabacterium* [1]). According to ORLA-JENSEN, the homofermentative, rod-shaped lactic acid bacteria, which are thermophilic, should be united in the genus *Thermobacterium*. The representatives of the genus *Streptobacterium* and the genus *Betabacterium* can be distinguished from each other on the basis of a number of characteristics.

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[1]) The genus *Microbacterium*, the representatives of which are catalase-positive, is left out of consideration here.

One of the differences between these genera is the ability of the *Betabacterium* species, contrary to the *Streptobacterium* species, to form gas and volatile acid from carbohydrates. Furthermore, ORLA-JENSEN has shown that the homofermentative *Streptobacterium* species are good salicin fermenters, while the heterofermentative *Betabacterium* species either have this ability only to a very small extent, or not at all. As a third useful characteristic should be mentioned that as a rule the *Streptobacterium* species grow very well in milk, whereas the *Betabacterium* species only develop very poorly in this medium, if at all.

As the isolated strains proved not to be thermophilic, they could not belong to the genus *Thermobacterium*. In order to determine whether they should be classified in the genus *Streptobacterium*, or in the genus *Betabacterium*, the strains of the above mentioned groups I, II and III were tested on their ability to form gas from glucose. For that purpose the various strains were inoculated in fermentation tubes according to STRUYK (28), which were filled with yeast autolysate [1]) with 2% glucose. Experience has shown that in these fermentation tubes even slight gas formation can be easily ascertained. For this reason they are most suitable for establishing the occurrence of the usually small quantities of carbon dioxide resulting from the sugar fermentation by the heterofermentative lactic acid bacteria.

Secondly, all strains were inoculated in sterile milk. It appeared that even those strains that grew rather well in the milk did not bring about coagulation or only after a very long incubation period. This changed, however, on addition of 10% yeast autolysate to the milk. Apparently the autolysate added the necessary accessory food substances to the poor winter milk.

Finally, all isolated strains were inoculated in a medium consisting of yeast autolysate with 2% salicin.

It can be said at once that the results of these tests were in so far highly satisfactory that all strains collected in one separate provisional group showed a quite homogenous behaviour. All results are collected

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[1]) The yeast autolysate is prepared by autolyzing 1 kg yeast („koningsgist" made by the „Nederlandsche Gist- en Spiritusfabriek" at Delft), in 1 l water during 24 hours at a temperature of 50° C, whereupon the mass is boiled and filtered. Then the pH was adjusted to about 6 with the aid of dilute sodium hydroxide. The liquid is then boiled once more and filtered again. The nitrogen content in the autolysate thus obtained approximates 1%.

in Table 1 which also indicates whether the examined strains formed dextran and capsules from saccharose.

Table 1.

Different characters of the lactic acid bacteria of the groups I, II, III.

bacteria of	dextran production from saccharose	formation of capsules	gas production from glucose	curdling of milk + 10% autolysate	fermentation of salicine
group I. .	—	—	—	+	+
group II .	+	—	—	—	+
group III .	+	+	+	—	—

These experiments lead to the conclusion that both the strains of group I and group II belong to the genus *Streptobacterium*, although the bacteria of group II do not grow in milk. In this connection it should be remarked that ORLA-JENSEN also admits that certain *Streptobacterium* strains miss the power to develop in milk.

To the contrary the bacteria of group III do unmistakably belong to the genus *Betabacterium*.

The question now arises whether the isolated *Streptobacterium* species and the *Betabacterium* species can be identified with species already described. To begin with the *Streptobacterium* species, we distinguish since the work of ORLA-JENSEN in this genus two species, *viz.*, *Streptobacterium casei* and *Streptobacterium plantarum* (as for the third species mentioned by BERGEY, *viz.*, *Lactobacillus leichmannii*, see below). The first species, which ORLA-JENSEN used to isolate from cheese and milk products, as a rule prefers lactose to maltose and saccharose, and only rarely ferments raffinose, inulin and pentoses. The second species, a typical saprophyte occurring on vegetable products, generally prefers maltose and saccharose to lactose, and frequently ferments raffinose, inulin and pentoses.

PEDERSON (21) who made an extensive study of *Streptobacterium plantarum* has come to the conclusion that the majority of the strains form acid in glucose, galactose, fructose, mannose, maltose, saccharose, lactose, raffinose, arabinose, and salicin, to a lesser extent in sorbitol, mannitol, dextrin, glycerol and xylose, and as a rule not at all in rhamnose, starch and inulin. This satisfactorily conforms with ORLA-

JENSEN's data, though the latter attributes to *Streptobacterium plantarum* ample ability to ferment inulin (*Streptobacterium casei* is inulin–), while PEDERSON denies this characteristic to this species. In the meantime HORNBOSTEL's experiments (11) have clearly demonstrated the existence of various intermediate forms between the two species under discussion.

The ability of our strains to ferment various sugars was tested in the following way. 5 cc of a 4% solution of the various sugars in distilled water were pipetted, after sterilisation, to 5 cc sterile yeast autolysate. The tubes were then inoculated with young pure cultures, and incubated during a fortnight at a temperature of 30° C. At the expiration of this period 5 cc of the medium was diluted with distilled water to 50 cc, boiled softly to remove any carbon dioxide present, and titrated with 0.1 n NaOH (with phenolphthalein as an indicator). The original NaOH consumption of the medium was subtracted, and the acidity then calculated on the basis of cc of normal acid on 100 cc of the medium.

1. bacteria of group I.

The results obtained in the experiments on the fermentation of the various sugars, sugar-alcohols and glucosides by the *Streptobacterium* strains of group I, are reproduced in Table 2.

Table 2.

Acid production from different carbon sources by the lactic acid bacteria of group I.

Strain	A1	B2	C1	C2	C3	C4	C5	E1	H1
Glucose . . .	15.0	11.8	12.5	18.3	19.7	15.2	11.1	13.0	14.3
Galactose . .	11.8	9.8	9.5	16.9	12.6	13.6	8.2	11.0	11.8
Fructose. . .	16.7	14.5	15.0	17.5	19.7	17.5	13.1	15.2	11.6
Mannose. . .	15.8	13.4	12.9	18.9	15.1	17.1	9.6	13.7	15.3
Maltose . . .	14.5	11.8	12.1	16.5	16.4	18.5	10.4	14.0	14.6
Saccharose. .	13.7	11.1	11.1	11.4	15.6	18.5	9.4	13.7	14.2
Lactose . . .	12.5	10.4	10.6	16.7	15.3	13.4	9.8	12.0	12.4
Raffinose . .	12.4	11.7	8.8	15.5	15.2	16.8	12.2	13.0	11.3
Inulin . . .	—	—	—	—	—	—	—	—	—
Dextrin . . .	5.7	4.3	4.0	4.5	4.7	5.6	4.5	3.7	3.0
Starch. . . .	—	—	—	—	—	—	—	1.3	1.0
Arabinose . .	—	—	—	—	—	—	—	—	—
Xylose . . .	—	—	—	—	—	—	—	—	—
Glycerol. . .	—	—	—	—	—	—	—	—	—
Mannitol . .	7.8	6.3	4.1	8.8	7.8	8.2	6.2	7.7	6.6
Salicin . . .	11.3	10.2	10.1	14.4	13.7	13.3	9.6	11.1	11.3

The agreement between the various strains is such, that they can be considered to belong to one and the same species. A comparison of ORLA-JENSEN's data with the results obtained by us leads to the conclusion that the strains we studied all belong to the species *Streptobacterium casei*. In our case, however, there was no indication of this species preferring lactose to maltose and saccharose, as stated by ORLA-JENSEN. A more remarkable fact is that our strains of *Streptobacterium casei* all proved fully capable of fermenting raffinose, which characteristic is not attributed to this species by ORLA-JENSEN, nor by HORNBOSTEL.

On first consideration it seems surprising that we so frequently isolated *Streptobacterium casei*, since our starting material was of vegetable origin, whereas ORLA-JENSEN reports to have isolated this species exclusively from milk and milk products. In this connection it should be observed, however, that ELEMA (4) repeatedly isolated *Streptobacterium casei* from potato pulp, thus also from vegetable material.

We must add that the strains C2, C5 and E1 form a small quantity of dextran from saccharose. This property is, however, only slighty developed.

2. bacteria of group II.

Table 3 shows the degree to which the various sugars, sugar-alcohols, and glucosides are attacked by the strongly dextran-forming *Streptobacterium* strains of group II.

On examining the results more closely, one is at once struck by the fact that these strains, which hardly differ, do not ferment lactose. This is in accordance with our previous statement that these four strains hardly develop in milk.

It is clear that also these strains should be considered as belonging to one species of the genus *Streptobacterium*. This species distinguishes itself from the *Streptobacterium* species described by ORLA-JENSEN by its inability to ferment lactose, and by its striking characteristic of forming dextran from saccharose. The weak ability to ferment galactose and maltose should further be noted, as both *Streptobacterium casei* and *Streptobacterium plantarum* usually ferment these sugars vigorously.

On the basis of the classification accepted by BERGEY, the rod-shaped lactic acid bacterium in question should be considered to belong to the species *Lactobacillus leichmannii* Bergey et al. HENNEBERG (10) has

Table 3.

Acid production from different carbon sources by the lactic acid bacteria of group II.

Strain	B1	F1	G1	H2
Glucose . . .	15.7	17.3	18.4	13.5
Galactose . .	—	—	2.1	—
Fructose. . .	17.2	18.9	18.6	15.0
Mannose. . .	16.1	16.9	16.3	14.1
Maltose . . .	4.0	4.5	3.5	2.0
Saccharose. .	15.1	14.6	14.7	13.4
Lactose . . .	—	—	—	—
Raffinose . .	—	—	2.9	3.5
Inulin. . . .	15.1	16.2	15.9	15.1
Dextrin . . .	0.7	—	1.4	1.1
Starch. . . .	—	—	—	—
Arabinose . .	—	—	—	—
Xylose . . .	—	—	—	—
Glycerol. . .	—	—	—	—
Mannitol . .	7.6	6.1	7.0	5.3
Salicin . . .	11.5	10.7	12.0	10.1

defined this species as *Bacillus leichmanni I*, which is probably identical with the species *Bacillus leichmanni II*, simultaneously described by him. This rod-shaped, homofermentative lactic acid bacterium, isolated by HENNEBERG from milk and yeast, is considered by BERGEY as well-nigh identical with *Thermobacterium Delbrücki*, with the exception of its having a lower temperature optimum (36° C.). Acid is produced from the following sugars: glucose, fructose, maltose, saccharose and trehalose. Small quantities of acid are formed from galactose, mannitol and α-methylglucoside. Lactose, raffinose, arabinose, rhamnose, dextrin and inulin are not fermented at all. HENNEBERG does not make any mention of the ability of this species to form dextran from saccharose.

In my opinion, there is every reason not to identify the *Streptobacterium* species isolated by me with *Lactobacillus leichmannii* Bergey et al. As far as HENNEBERG's data regarding the fermentability or infermentability of certain sugars, enable us to make a comparison, our *Streptobacterium* species differs from *Bacillus leichmanni I* by the weak fermentation of maltose, and by its being a strong inulin fermenter; principally, however, by its ability to form dextran in considerable quantities from saccharose. These points make it clear that there is also

no reason in defining it as a variety of *Lactobacillus leichmannii*, or rather of *Streptobacterium Leichmannii*, if this indeed should be accepted as a separate species.

Nevertheless it is clearly indicated to differentiate between our species and the two species — *Streptobacterium casei* and *Streptobacterium plantarum* — of ORLA-JENSEN. I should like to propose the name of *Streptobacterium dextranicum* for the new species, which name is chosen in order to call special attention to the pronounced ability of this homofermentative, rod-shaped lactic acid bacterium to form dextran from saccharose.

A more detailed description of the new species is given below:

Morphology: immotile, non-spore-forming, gram-positive small rods, usually 0.7 × (2—5) μ, occurring singly or in short chains.

Fig. 6. *Streptobacterium dextranicum* nov. spec., strain H2, on yeast-agar with 10% saccharose. × 0.5.

On yeast-agar with 2% glucose and 2% chalk at 30° C.: acid-forming colonies, 1 mm in diameter after 2 days, 2 mm after 7 days.

On yeast-agar with 10% saccharose at 30° C.: large slimy colonies which reach a diameter of 3 mm after a fortnight (Cf. fig. 6) and show a marked inclination to confluence. In microscopical preparations no capsule-formation has been observed.

On yeast-gelatine with 10% saccharose at 22° C.: large, drop-shaped colonies that develop to 2 mm in diameter after 5 days. No liquefaction of the gelatine.

Catalase-negative.

Litmus milk: no formation of acid and no coagulation.

Yeast-water with 2% glucose: turbid.

Yeast-water with 2% saccharose: turbid in the beginning: after a few days markedly slimy on account of dextran production. Finally the medium becomes strongly viscous and wholly untransparent.

Strong acid production in glucose, fructose, mannose, saccharose, inulin and salicin. Weak acid production in mannitol; very weak acid

production in galactose, maltose, raffinose, and dextrin. No acid production in lactose, starch, arabinose, xylose and glycerol.

Homofermentative lactic acid fermentation in the sense of KLUYVER and DONKER (12).

Optimum temperature 30—35° C. Maximum temperature 40° C.

The ability of this species to form dextran from saccharose is very pronounced. It should be explicitly stated that this polysaccharide, just as in the case of the dextran-forming cocci, is never formed from any other sugar.

3. bacteria of group III.

As stated before, the bacteria of group III belong to the genus *Betabacterium*. The results obtained in the investigation on the fermentation of various sugars, sugar-alcohols and glucosides, are collected in Table 4.

Table 4.

Acid production from different carbon sources by the lactic acid bacteria of group III, and by *Betabacterium vermiforme*.

Strain	C6	E2	F2	G2	H3	*Betabacterium vermiforme* (after MAYER)
Glucose . . .	11.2	12.1	12.8	10.5	10.5	9.5
Galactose . .	10.8	11.6	13.3	9.4	10.0	9.6
Fructose. . .	7.9	9.0	10.2	7.7	8.4	8.2
Mannose. . .	—	—	—	—	—	—
Maltose . . .	9.9	10.6	12.0	6.1	9.9	8.6
Saccharose. .	7.5	10.1	7.4	9.8	5.8	9.1
Lactose . . .	—	—	—	—	—	1.5
Raffinose . .	9.4	10.7	10.5	8.9	5.6	
Inulin. . . .	—	—	—	—	—	—
Dextrin . . .	—	—	—		—	—
Starch. . . .	—	—	—	—	—	—
Arabinose . .	29.2	29.6	32.3	23.8	25.6	21.9
Xylose . . .	27.4	28.1	31.0	24.2	24.7	21.5
Glycerol. . .	—	—	—	—	—	—
Mannitol . .	—	—	—	—	—	—
Salicin . . .	—	—	—	—	—	1.3

The five strains of group III again show a very satisfactory homogenity in behaviour towards the various carbon sources, and therefore may be deemed to belong to one and the same species.

The question thus arises whether there is reason to identify our strains with one of the earlier described *Betabacterium* species. In this connection our attention was at once drawn to *Betabacterium vermiforme* (Ward) Mayer for which species MAYER (18) has shown that it is characterized amongst others by its ability to form dextran from saccharose, an observation which quite recently has been fully corroborated by DAKER and STACEY (3) [1]).

An identification of our strains with *Betabacterium vermiforme* was the more tempting since both WARD (29) and MAYER give reproductions of encapsulated rods which greatly resemble the formations occurring in our cultures on saccharose-containing media.

In Table 4 we have included the results obtained by MAYER in his study of the behaviour of *Betabacterium vermiforme* towards various carbon sources. It is clear that also these data are quite in accordance with the suggested identity.

In this connection it seems desirable to give here some more particulars regarding *Betabacterium vermiforme*.

We may recall here then that this organism has been first isolated by WARD in 1892 as one of the constituents of the „ginger-beer plant" It was described by this author under the name of *Bacterium vermiforme*. WARD duly recognized that it did belong to the group of the lactic acid bacteria. As has already been remarked, a few years later WARD encountered *Bacterium vermiforme* for a second time when studying the organisms present in an excrescence of a sugar cane plant from Madagascar.

In later years the bacterium has been extensively studied by MAYER who isolated it as one of the two essential constituents of the so-called „Tibi"-grains. MAYER in the first place made observations which proved that the bacterium had all the properties characteristic of the genus *Betabacterium* Orla-Jensen.

A few more of the principal results obtained by MAYER in his investigation will be briefly summarized here.

On plating a pure culture of *Betabacterium vermiforme* on glucose-yeast-agar after a certain period another type of colony appeared next to the original rough colonies. In the long run, however, it proved impossible to make a closer study of this phenomenon on aerobic plates, as after some stages on these plates, all growth ceased. This difficulty

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[1]) DAKER and STACEY mutilate the name *Betabacterium vermiforme* (Ward) Mayer by writing *Betabacterium vermiformé* (Ward-Meyer).

could be overcome by cultivating the bacterium in question under strongly reduced oxygen tension, instead of under aerobic conditions.

The dissociation that occurred under these conditions could now be closely followed. It appeared that on the nutrient medium, besides the rough, flat-lens-shaped colonies with irregularly crenated edges (R-type), also variations occurred, mainly in the form of thin, flat, very extensive colonies (O-type). Besides these also round, or somewhat irregularly formed colonies, with a smooth, shiny surface (S-type) occurred.

MAYER now made the interesting observation that only the R-type (and in some cases a still coarser R'-type), forms dextran from saccharose, in contradistinction to all other types of colonies, among which O and S. So we have here to do with the remarkable fact of dissociates showing constant differences also from a point of view of metabolism (+ or — dextran-formation).

It was now most gratifying that the strains of group III showed on the whole the same behaviour as described by MAYER for *Betabacterium vermiforme*. The strains had been first streaked a few times on plates of yeast-agar with 10% saccharose, or yeast-gelatine with 10% saccharose, in order to obtain pure cultures. Hereupon they were streaked on a saccharose-free nutrient medium on which no dextran could be formed, in view of the possibility that the dextran-forming colonies still might contain contaminating germs. However, when cultivating on yeast-agar with 2% glucose under aerobic conditions, no growth at all occurred. Like MAYER, however, I obtained satisfactory results by cultivating these plates in an atmosphere of hydrogen.

After the strains had been cultivated for some time on yeast-agar with 2% glucose, also a dissociation into two types occurred. These types conformed to the types R and S, as described in detail by MAYER. The O-type only appeared quite sporadically on the plates. Also in my case the R-type possessed the ability to form dextran from saccharose, whilst the S-type was not capable to do so. The dissociation also took place on yeast-agar and yeast-gelatine with 10% saccharose. This dissociation can, of course, quite easily be observed, as non-dextran-forming colonies appear on the plates besides the dextran-forming colonies. When streaked on yeast-agar with 2% glucose, the dextran-forming colonies showed R-type colonies in excess· on the other hand, when non-dextran-forming colonies were cultivated on the said medium, the S-colonies prevailed.

On summarizing all foregoing observations made on the strains of group III is seems safe to conclude to the identity of these strains with *Betabacterium vermiforme.*

A few remarks will still be made concerning some properties of *Betabacterium vermiforme.*

MAYER does not discuss the question why *Betabacterium vermiforme* grows well on saccharose-containing media under aerobic conditions, but not on glucose-containing nutrient media. In my opinion this phenomenon will be due to the fact that *Betabacterium vermiforme* is adapted to low oxygen tensions, and succeeds in securing the necessary anaerobiosis by the formation of capsules from saccharose which diminish the rate of penetration of oxygen into the cells. As no dextran is formed on the glucose-containing nutrient media, there is no question here of a protecting action, and a satisfactory development only results when the cultivation is done under strongly reduced oxygen tension.

In the meantime, it is not advisable to maintain the strains on a medium consisting of yeast-agar with 10% saccharose, although, as already observed, *Betabacterium vermiforme* grows very well on this medium. Experience shows, however, that after a relatively short period (within a month) the pure cultures in tubes with yeast-agar with 10% saccharose are no longer transferrable. Obviously, in the long run the oxygen still unfavourably affects the viability of the cells cultivated on this medium.

On the other hand, the isolated strains of *Betabacterium vermiforme* can successfully be kept in culture tubes with yeast-agar with 2% glucose, if initially cultivated under anaerobic conditions. It is also quite feasible to grow the bacterium in question in liquid cultures of yeast-water with 2% glucose. Finally, also yeast-gelatine with 10% saccharose is eminently suitable for the maintenance of the strains in contrast to the behaviour of the cultures on yeast-agar with 10% saccharose. This divergence may be explained by the fact that remarkably the strains penetrate rather deeply into the gelatine — without, however, causing liquefaction — while in the tubes with yeast-agar with 10% saccharose, the growth only takes place on the agar surface. The latter bacteria are, therefore, apparently more exposed to the harmful influence of the oxygen.

6. DISCUSSION OF RESULTS.

On reviewing the results described in the foregoing chapters we may conclude that the study of the bacteria present in the „Froschlaich"-grains under investigation has led to the isolation of rod-shaped lactic acid bacteria of three different types. One of these could be identified with *Streptobacterium casei* Orla-Jensen. Its significance for the forma-

tion of the grains is most doubtful, since the greater part of the pure cultures never produced any mucus, neither on saccharose-containing media, nor on any other medium tried.

The second type proved to be a new representative of the genus *Streptobacterium* to which the name *Streptobacterium dextranicum* was given, because it had the property — unknown for the *Streptobacterium* species described until now — to form large quantities of dextran out of saccharose. Since, however, the mucus produced by this species always had a very soft consistency, and since encapsulated bacteria were never observed herein, its importance for the formation of the hard and tough „Froschlaich"-grains can only be of a secondary nature. This does not mean that the occurrence of *Streptobacterium dextranicum* in the beet juice of a sugar-factory will not give rise to serious losses on account of the ample production of dextran from saccharose.

The third type of lactic acid bacteria isolated which could be identified with *Betabacterium vermiforme* (Ward) Mayer proved to be the chief organism responsible for the „Froschlaich"-formation. The pure culture on saccharose-containing media showed rods with very heavy capsules of exactly the same type as found in the original grains.

This result is undoubtedly remarkable from several points of view.

In the first place it should be noted that until now *Betabacterium vermiforme* has only been isolated from more or less exotic products, *viz.*, from the „ginger-beer plant" and „the excrescence of a cane sugar plant from Madagascar" by Ward, and from „tibi-grains" by Mayer. The occurrence of *Betabacterium vermiforme* in Dutch soil or as a saprophyte on beets grown in Holland has, therefore, a more or less startling character.

Secondly the literature on „Froschlaich" as appearing in sugar-factories leaves no doubt that in by far the majority of the cases investigated the said formation, if caused by rod-shaped bacteria, is always due to the action of spore-forming rods more or less closely related to *Bacillus vulgatus*. In such cases, however, chemical analysis reveals that the „Froschlaich" consists of laevulan.

It seems, therefore, quite noteworthy that in the case under investigation we have met with an exception to this rule: the causative organism being a sporeless lactic acid rod, the mucus formed having the composition of dextran, exactly as in the case of the most common „Froschlaich", caused by the coccus-shaped *Leuconostoc mesenterioides*.

To these considerations only one remark should still be added. It

seems very probable that a „Froschlaich" of the same type as studied by us has also been described by KOCH and HOSAEUS in their publication already cited (13). This may be concluded from the drawings of the capsulated bacteria as observed by these authors, which drawings show a striking resemblance to the pictures given by WARD in his classical paper on *Betabacterium vermiforme*. However no certainty can be given, since KOCH and HOSAEUS have been unable to isolate the bacterium out of the „Froschlaich".

Summary.

From a „Froschlaich" formed in a sugar-factory, the responsible organism was isolated. This organism could be identified with the dextran-forming, heterofermentative, rod-shaped lactic acid bacterium, *Betabacterium vermiforme*, a bacterium first described by WARD as one of the constituents of the „ginger-beer plant" and recently recognized by MAYER (18) as being also one of the components of the „tibi"-consortium. Thus it has been shown that the formation of the „Froschlaich" in sugar factories is not always due to *Leuconostoc mesenterioides* or to *Bacillus vulgatus*, but can also be caused by *Betabacterium vermiforme*.

A hitherto unknown species of the genus *Streptobacterium*, was found to be an accompanying organism. This rod-shaped, homofermentative, dextran-forming bacterium has been described in detail as the species *Streptobacterium dextranicum* nov. spec.

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(From the Microbiological Laboratory, Agricultural University, Wageningen).

THE FORMATION OF ACETIC ACID FROM CARBON DIOXIDE AND HYDROGEN BY ANAEROBIC SPORE-FORMING BACTERIA

by

K. T. WIERINGA

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In a previous paper (6) I described the bacteria which are able to synthesize acetic acid from hydrogen and carbon dioxide. Those investigations had been started by SÖHNGEN in the last period of his life as a sequel to his earlier work (5) on the methane-bacteria. Aiming to arrive at a pure culture of these organisms SÖHNGEN chose tap water with mineral salts and a gas-mixture of H_2 and CO_2, presumably a culture medium of highly selective qualities. In fact SÖHNGEN noted the disappearance of 1191 cc of H_2 and 300 cc CO_2 in a fortnight. In this period 285 cc of CH_4 had been formed. As, theoretically, one volume of CH_4 is formed from 4 volumes of H_2 and one volume of CO_2, 51 cc of H_2 and 15 cc of CO_2 are not accounted for by the above reaction.

In order to study this process more in detail, it was necessary to accelerate it. This increase in rate could be brought about by different means. One of these consisted in the construction of a culture apparatus well fitted for the purpose of gas absorption. A culture flask with a capacity of 2.5 l was described and pictured in fig. 1 and 2 of my previous paper, and the composition of a more suitable culture medium was given. It contained a few inorganic salts (0.1% K_2HPO_4, 0.05% $MgSO_4$, and 0.2% $(NH_4)_2SO_4$) solved in an extract of fat-free ditch mud, 50 g of mud (after treatment with benzine) being shaken overnight with a 6% $NaHCO_3$ solution and filtered through a filter candle (Leune no. 7) directly into the flask. Finally 1 cc of a sterile saturated Na_2S-solution and 5 mg $FeSO_4$ are added per l. In this way the medium is poised to maintain a low redox level. Also the presence of colloidal FeS seems to be favourable, as has also been stated by LIESKE c.s. in experiments on reduction of CO and CO_2 to CH_4 (1,3). Inoculation of

1 cc or less of a crude culture in this medium will get a subculture to start within a week generally. Sometimes, however, the incubation period is a fortnight or more.

Now it was found that subcultures of this type sometimes may absorb large amounts of hydrogen without any formation of CH_4. Microscopical examination of such cultures shows the presence of club-shaped spore-bearing bacilli. Inoculation of 10 cc of the pasteurised culture in a new flask gives a subculture which appears to contain hardly any but the latter bacilli. It is relatively easy to obtain a pure culture of these organisms by sowing a pasteurised culture on agar plates. Initially the agar was made up of the usual tap water agar, to which, next to mineral salts, $^1/_3$ of sterile filtered mud extract had been added; these plates have to be placed in an exsiccator filled with hydrogen. Subsequently malt extract agar was substituted for tap water agar; this medium gives a more abundant growth. Moreover the sugar in this alkaline medium warrants a low redox potential, and forms a good source of energy for these organisms. That is why it is possible to dispense with the hydrogen atmosphere, and to cultivate the bacteria in deep layers in ordinary culture tubes. Pure colonies of the bacilli described above will grow abundantly in a stoppered flask filled with a liquid medium consisting of 2 parts of malt extract and 1 part of $NaHCO_3$-mud extract. No gas is formed in this medium. After some days the dense turbidity caused by bacterial growth will settle, leaving the liquid quite clear and transparent. Inoculation of the sediment of such a culture into a flask with mud extract and H_2 will show, usually within 2 or 3 days, a very rapid disappearance of the H_2.

The rate of the assimilation of hydrogen was measured manometrically. To this end manometers were fastened to a column placed centrally upon the disc and connected to the side-tube of the culture flasks. In active cultures a nearly total vacuum will occur in 48 hours as shown in fig. 1. The curve runs down steeply at first and later goes nearly parallel to the time axis. In the case of crude cultures the initial course of the process is the same. The endpoint, however, will be reached at a much higher pressure of the gas, one part of CH_4 being formed when 4 parts of H_2 have been consumed. So the process stops when the gas pressure has decreased to $^1/_4$ of the original.

As can be taken from fig. 2 a simple mathematical relation exists between the rate of assimilation of the hydrogen and the pressure of this gas. If the logarithms of the pressure are plotted against the time a straight line results. Mathematically this means that $-\log P_t = Kt$

$-\log P_o$, when P_o = pressure at a time t = o and P_t = pressure at a time = t. The reaction constant $K = \frac{1}{t} \log \frac{P_0}{P_t}$. In the units chosen here (*viz.*, mm of Hg and hours) K = 0.0265. As $[H_2]$ (= conc. of hydrogen molecules) may be substituted for P, $-\log [H_2] = Kt - -\log [H_2]_0$ and on differentiation

$$\frac{-d [H_2]}{dt} = K.$$

Supposing the gas to be pure hydrogen, this means that at any moment the number of H_2 molecules used for synthesis of acetic acid is proportional to the number of molecules present. This supposition holds fairly true, but at the end of the process, when most of the hydrogen has been assimilated, the curve shows a distinct deviation from the straight line as can be seen in fig. 2. This is caused by the vapour pressure and the pressure of CO_2, which are then relatively high.

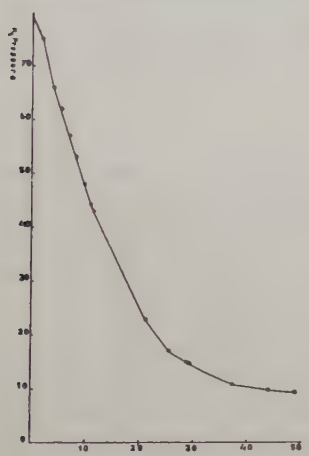

Fig. 1. Relation of H_2-pressure and H_2-absorption.

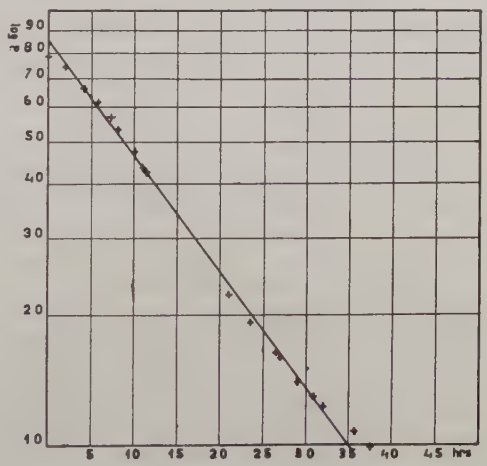

Fig. 2. Relation of H_2-pressure and H_2-absorption.

As stated in my previous paper H_2 and CO_2 are combined by these bacteria to acetic acid:

$$4H_2 + 2CO_2 = CH_3COOH + 2H_2O,$$

or, when $NaHCO_3$ is used as a source of CO_2, as was the case in these experiments:

$$3NaHCO_3 + 4H_2 = CH_3COONa + Na_2CO_3 + 4H_2O.$$

In the latter case it is clear that the liquid must become more and more alkaline in spite of the acetic acid formed. Carbon dioxide has to be added in order to allow the process to go on. HCl may be added to the same purpose. As long as Na_2CO_3 is present the culture will continue to absorb H_2. As an example I mention a culture inoculated on 29-VIII-'38. When the pH (measured by the glass electrode method) had gone up to 9.56, 5 cc of concentrated HCl were added, bringing the pH down to 9.12. The next day again 5 cc of HCl were added. The pH was now lowered to 7.80. Table 1 shows the increase of H_2 absorbtion effected by these additions. The total alkali and $NaHCO_3$ were determined in 5 cc of the liquid.

Table 1.

	H_2 assimilated	pH	HCl added	Total alkali in 5 cc of the liquid	$NaHCO_3$
Sept. 7	400	—	—	—	—
,, 8	1560	—	—	—	—
,, 9	1230	—	—	—	—
,, 10	1300	—	—	—	—
,, 11	1320	—	—	—	—
,, 12	1190	—	—	—	—
,, 13	1000	—	—	—	—
,, 14	680	9.12	—	—	—
,, 15	500	—	—	—	—
,, 16	600	—	—	—	—
,, 17	450	9.56	5 cc	21.6	5.2
,, 17	—	9.12	5 cc	16.1	9.1
,, 18	1090	7.80	—	—	—
,, 19	940	—	—	—	—
,, 20	1130	—	—	—	—
,, 21	1000	—	—	—	—
,, 22	800	—	—	—	—
,, 23	220	9.65	—	7.0	0

When no CO_2 or H_2 is added the pH will go on rising until the culture stops taking up H_2. An example of this is given in Table 2.Initially the total alkali in 5 cc titrated 24.9 cc N/10, the $NaHCO_3$ taking 19.4 cc N/10; pH was 8.6. At the end the pH was as high as 10.4.

Table 2.

Date	Volumes of H_2 absorbed	Date	Volumes of H_2 absorbed
28–XI –'38	170 cc	5–XII–'38	890 cc
29.....	2120 ,,	6.....	1270 ,,
30.....	1170 ,,	7.....	1160 ,,
1–XII–'38	1300 ,,	8.....	1140 ,,
2.....	1280 ,,	9.....	1050 ,,
3.....	1200 ,,	10.....	80 ,,
4.....	1120 ,,	11.....	0 ,,

As the hydrogen ion concentration is a very important factor in these cultures a series of flasks was filled with mud extract, brought to different pH by addition of HCl or NaOH. In these flasks the assimilation of H_2 was measured during 42 days. The total alkali and bicarbonate alkali were measured before and after this period by titration of 5 cc of the solution. Table 3 gives the results of the experiments.

Table 3.

No.	Total alkali in 5 cc (of solution)	$NaHCO_3$ in 5 cc (of solution)	pH	Total alkali in 5 cc (of solution)	$NaHCO_3$ in 5 cc (of solution)	pH	H_2 assimilated (l)
1	19.9	19.6	7.18	—	—	—	0.0
2	24.6	23.2	7.6	—	—	7.9	0.360
3	27.9	25.3	8.1	18.1	1.8	10.1	19.620
4	27.3	22.0	8.6	20.2	4.1	10.0	14.040
5	28.3	19.6	9.0	23.6	6.6	10.1	9.360
6	34.1	10.2	9.5	31.8	1.8	10.2	6.970
7	42.3	4.6	10.0	—	—	10.0	0.490
8	45.7	1.0	10.5	—	—	10.5	0.0

From this experiment it follows that the optimal pH is in the neighbourhood of 8 or 8.5. At a pH of 7 or 10.5 the process will not start at all. The pH reached in the end is not appreciably above 10. However, the behaviour of some of these cultures appeared to be at variance with the expectation. This might be ascribed either to the variability in the bacteria or to the culture media. None of the cultures of Table 3 was as vigorous as could be hoped. The highest figure of H_2 taken up in 1

day was 1320 cc, whilst other cultures used 1500 cc or even more in the same time.

From fig. 2 it follows that the best conditions for the hydrogen assimilation may be expected when the gas pressure is kept constant. To avoid too high a pH, CO_2 has to be supplied along with H_2 without interrupting the rotation of the flasks. To this end the gas inlet of a culture flask was connected to a hydrogen generator, the gas current passing through a gasmeter. A bell jar (*b*) plunging in an oil-bath (*a*) was interposed between the gasmeter and the flask (see fig. 3). The central tube (*c*) of the oil bath was connected to the culture flask. The oil bath carried a ball-bearing (*d*) by which the bell jar could rotate. This apparatus was placed in a central column upon the disc. The gasmeter may be connected alternatively with a H_2- and a CO_2-generator. An experiment carried out in this way follows in Table 4.

Fig. 3. Revolving gas connection.

Table 4.

Time in days	Volumes of gas recorded by the gasmeter	
	H_2	CO_2
1	4510	—
2	2660	980
3	2320	1050
4	—	3955
7	7595	1470
9	5160	960
10	2480	—
11	—	3780
Total	24725	12195

In these 11 days more than 2 l a day of H_2 had been taken up, the highest amount being 4.5 l. At the end of the period the pH was 7.96. The total alkali of the solution titrated 13.35 cc N/10, all of it being present as $NaHCO_3$. The alternative administration of H_2 and CO_2 not being ideal, maximal achievements cannot be expected under these circumstances.

Now in attempting to classify the organism described here, I will repeat its most prominent characters determined thus far. Morphologically the organism is an obligately anaerobic spore-forming motile schyzomycete, producing plectridia at sporulation. Among the biological characteristics the formation of acetic acid can be placed in the foreground. This acid is not merely formed from H_2 and CO_2, but also from sugar. In a mixture of 2 parts of malt extract to 1 part of mud extract 0.3% to 0.35% of acetic acid had been formed in a week. The acid has been determined by destillation. Before acidifying, the carbon dioxide was eliminated by addition of sufficient baryta. After filtering through paper 50 cc of the filtrate were acidified with H_2SO_4 and diluted to 400 cc with distilled water. This solution was distilled using the method of KNETEMANN (2). An organism with these characters could not be found in literature; so it must be given a name. Its morphology makes it evident, that it is to be classified in the genus *Clostridium* Prazmowski. As a species-name I propose *aceticum*. The organism will then be called *Clostridium aceticum* n.sp. This name seems logical and in analogy with *Clostridium butyricum* occurring in the same genus.

Now it will be tried to answer the question: „is there a growth promoting substance present in the mud extract, and if so, what is its nature". It has been shown, that in a mineral solution with an atmosphere of H_2 and of CO_2 no appreciable growth is possible without mud or mud extract in the presence of alkali such as $NaHCO_3$. But even in cultures in malt extract the addition of the mud extract is very important. It is easy to show, that this is not simply caused by the alkalising action of the $NaHCO_3$. When cultures in the mud extract - malt extract mixture are compared with those in malt extract alkalized with $NaHCO_3$, growth is much more rapid in the former. In a number of such experiments the growth of *Cl. aceticum* was measured by determining the turbidity with the Pulfrich turbidity meter. Small samples of the culture medium were taken out of the flasks and replaced by N_2. The flasks were shaken during 2-3 minutes before sampling. An example of such an experiment follows in Table 5.

Table 5.

Time in hrs.	Turbidity in	
	$NaHCO_3$	mud extract
5	66	10
$22\frac{1}{2}$	133	431
27	156	631
29	186	2552

Other experiments gave similar results. So the extract was proven to contain one or more substances stimulating the growth of *Cl. aceticum*. These substances were not minerals, as was shown by adding ashes of the mud extract to cultures in malt extract as well as in synthetic media.

As was stated before, sterilising the mud extract will impede its stimulating action. One reason is the desintegration of $NaHCO_3$ by heat which results in too high a pH. On the other hand organic materials in an alkaline medium may desintegrate by sterilisation. In order to decide between various possibilities the bicarbonate mud extract was dialysed through cellophane in streaming tap water. Preliminary experiments had shown, that during dialysis a part of the activety was lost. Therefore a second portion was dialysed after acidifying the mud extract. The dialysis was continued until neutral reaction (2 days). Now half of either portion was boiled during 10 minutes and afterwards filled up to the original volume. With these 4 portions the following 8 solutions were made after addition of 2 parts of malt extract to each of them.

I. Alkaline mud extract dialysed
- *a*. Not boiled + 4% Na_2HPO_4, pH = 8.08, turbidity = 4
- *b*. ,, ,, + 1% $NaHCO_3$, pH = 8.40, ,, = 4
- *c*. Boiled + 4% Na_2HPO_4, pH = 8.30, ,, = 4
- *d*. ,, + 1% $NaHCO_3$, pH = 8.24, ,, = 6

II. Acidified mud extract dialysed
- *a*. Not boiled + 4% Na_2HPO_4, pH = 8.08, turbidity = 6
- *b*. ,, ,, + 1% $NaHCO_3$, pH = 8.22, ,, = 3
- *c*. Boiled + 4% Na_2HPO_4, pH = 8.28, ,, = 14
- *d*. ,, + 1% $NaHCO_3$, pH = 8.28, ,, = 116

Finally these solutions were sterilised by filtering, and inoculated with a fresh culture of *Cl. aceticum*. At different times the turbidity

and the acetic acid produced were measured. These were as follows in Table 6.

Table 6.

No. of culture	Turbidity after hours		Acetic acid in 50 cc after hours	
	31	35	45	93
I*a*	27	138	3.9	12.8
b	167	2117	8.4	—
c	90	175	4.7	12.5
d	82	2462	8.4	15
II*a*	23	126	4.7	15.4
b	230	2515	9.7	15.6
c	212	1894	8.4	15.7
d	259	2572	9.3	16.1

In another experiment addition to the malt extract of dialysed and not dialysed mud extract were compared with the mere addition of $NaHCO_3$.

Also the effect of dialysing the malt extract was studied; in this case mud extract and 1% glucose were added. Once more sodium bicarbonate and phosphate were compared as buffers. In these solutions only the turbidity has been measured (see Table 7).

Table 7.

	Turbidity after hours					
	0	22	23.5	27	30	32
malt extract + mud extract	7.4	138	180	251	1400	2077
malt extract + dial. mud extract + 1% $NaHCO_3$	72.9	137	152	171	635	710
malt extract + dial. mud extract + 4% Na_2HPO_4	24.6	88	98	173	481	688
dial. malt extract + mud extract + 1% glucose.	12.1	19.3	—	45.3	48	53.3
malt extract + $NaHCO_3$.	24.4	25.6	—	36	53	91

From these experiments it follows, that during dialysis of the mud extract part of its active substances are lost. Dialysing of slightly acidified extract is but little less harmful than when the extract is left alkaline. During dialysis of the malt extract important substances — presumably mineral salts and sugars — are lost. The mere addition

of sugar is not sufficient to compensate the loss. Boiling of the neutral malt extract does not impede its activity. $NaHCO_3$ seems to be somewhat better as a buffering substance than Na_2HPO_4. As far as my experience goes, the active substance in the mud extract is organic in nature and has a rather small molecule. Perhaps aggregates of molecules are present, somewhat peptonised by alkali. Experiments on the influence of adding the dialysate to the dialysed fluid could not be made through the lack of a suitable dialysator, but they will be taken in the future.

In absorption experiments it was shown that active coal can absorbe all of the active principle from the mud extract, provided the extract has been neutralized beforehand. It is sufficient to add 5 g of active coal („noriet") to the extract, to shake for some minutes, and then to filter through paper. The filtrate, which is nearly colourless, is free from the specific growth promoting substances, which now occur upon the coal as was indicated by turbidity measurements. Coal thus treated may now be sterilized without losing its activity.

It was furthermore tried to concentrate the active substance by the chromatographic method (7). The mud extract was therefore filtered through various substances such as Al_2O_3, $CaCO_3$, kaolin and $CaSO_4$. Of all these substances only $CaSO_4$ retained the active principle, whilst in the other cases the filtrate was as active as the mud extract itself. When, however, the mud extract is filtered through a 10 cm column of $CaSO_4$, the filtrate, of a slightly brown colour, is nearly inactive. The upper 2 cm of the $CaSO_4$ have held back the bulk of the brown substances. In this layer the $NaHCO_3$ of the mud extract has acted upon the $CaSO_4$ to form $CaCO_3$. Instead of $CaSO_4$ other Ca-salts such as $CaCl_2$ and Ca-acetate can be used to concentrate the active substance by precipitation. When the precipitate, existing chiefly of $CaCO_3$, is treated with HCl a brown precipitate is formed again, as soon as the $CaCO_3$ has been transformed to $CaCl_2$ and the solution becomes acid. After centrifugation this precipitate can be taken up in $NaHCO_3$. This solution is as active as the normal mud extract. No further experiments were carried out to purify the substance or to determine its chemical composition. Nothing can as yet be stated as to the nature of its chemical action. It is not known whether it acts as a food substance or merely in a chemical or physical way *e.g.*, such as stabilising the redox potential at a more suitable level. Such a possibility was taken into consideration because it is known that certain humic substances readily absorb O in alkaline media (4).

Therefore some experiments were carried out in order to get an idea of the redox potentials in culture media to which different substances had been added. Simultaneously the pH was measured in these media by the glass-electrode method, and growth was estimated by a rough evaluation of the turbidity. In one experiment 5 solutions were compared:

1) 2 parts of malt extract + 1 part of mud extract
2) as 1 with 0.05% Na_2S
3) as 1 with 0.1 % Na_2S
4) as 1 with 0.5 % Na_2S
5) malt extract + $NaHCO_3$

The results of this experiment can be taken from fig. 4.

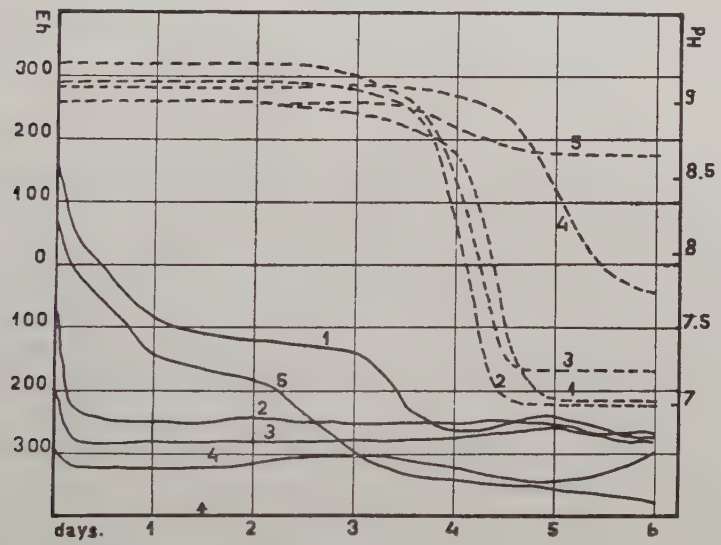

Fig. 4. Change of E_h (————) and pH (– – – – –) with time in various culture media inoculated at ↑.

After standing for 36 hrs in the incubator at 30° C. the culture media were inoculated. After a lapse of 28 hrs the first to become turbid was no. 3 and 3 hrs later no. 2 showed turbidity. Another 3 hrs later no. 1 and 4 became turbid, 2 and 3 being this time densely turbid. 76 hours after inoculation the culture in no. 2 and 3 had settled, leaving the solution clear. In no. 1 the clearing of the solution had proceeded half way down the flask, whilst no. 4 was quite turbid now. In no. 5 the medium became slightly turbid during the second day after the inoculation and remained so throughout the experiment.

From this experiment it can be concluded, that addition of Na_2S

stabilises the redox potential on a lower level. Low concentrations seem to be favourable whereas high concentrations (0.5%) are injurious. With the mere addition of $NaHCO_3$ to the malt extract the culture medium is but poorly poised, the redox potential decreasing throughout the experiment to a very low level. The glass-electrode potentials indicate, that growth goes along with acid production. No conclusion can be drawn from the experiment as to the real function of the mud extract. Perhaps a combination of $NaHCO_3$ and a redox stabiliser could have furnished more information. Such experiments were carried out using $NaHCO_3$ and thiourea added to malt extract. The latter substance, however, proved to be poisonous in the concentration used (0.1%), especially when no mud extract was present. I hope to resume this investigation at a later date.

Summary.

Further experiments on an anaerobic bacillus synthesising acetic acid from CO_2 and H_2 are described. The organism in question was classified as *Clostridium aceticum* n.sp. Acetic acid is also formed from sugar.

It was shown, that at any moment the number of H_2 molecules used for synthesis is proportional to the number of molecules present. Continuous provision with H_2 and CO_2 influences the rate of the process favourably. In such conditions a culture may absorb as much as 4.5 l of H_2 a day at 30° C.

The pH range of *Cl. aceticum* is between 7.5 and 10.5, the optimum being between 8 and 9.

A growth promoting substance is present in alkaline mud extract. This substance can be concentrated by means of absorption or precipitation. Its nature is still unknown.

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(Aus dem Centraalbureau voor Schimmelcultures, Baarn).

BESCHREIBUNG EINIGER NEUER PILZARTEN AUS DEM CENTRAALBUREAU VOOR SCHIMMELCULTURES, BAARN (NEDERLAND).

VI. MITTEILUNG

von

F. H. VAN BEYMA THOE KINGMA

(Eingegangen am 21 September 1940).

Emericellopsis terricola nov. gen. nov. spec.

Dieser Pilz wurde von Frl. N. SCHIERBEEK in Baarn aus der Erde isoliert. Besonders auf kohlehydrathaltigen Nährböden bildet der Pilz zahlreiche schwarze Perithezien, deren Asci Sporen enthalten, welche besonders auffallen durch die Anwesenheit mehrerer hyaliner Säume mit spitzen oder lappigen Ausläufern. Merkwürdigerweise isolierte Frl. SCHIERBEEK noch eine Varietät dieses Pilzes mit den gleichen Perithezien und Asci. Die Askosporen der Varietät besitzen dieselbe Grösse wie die Hauptform, weichen jedoch dadurch ab, dass die Säume nicht sternförmig oder lappig geschlitzt sind, sondern glatt.

Die systematische Stellung.

Bei näherer Betrachtung der Gattungen, welche zu den *Eurotiaceae* gehören, fallen in erster Reihe diejenigen Merkmale der Perithezien oder Askosporen auf, welche einigen Arten dieser Gattungen gemeinsam sind. So findet man die sogenannten „Hülle"-Zellen von *Aspergillus nidulans* Eidam zurück bei *Emericella variecolor* Berk., welche Art als Konidienform wieder einen *Aspergillus, A. stellatus* Curzi besitzt (4). So besitzen die Askosporen des uns hier beschäftigenden Pilzes ähnliche leistenförmige Vorsprünge, wie sie bei *Penicilliopsis clavariaeforme* Solms-Laub. auftreten (7), zugleicher Zeit aber geht von diesen Vorsprüngen je ein flügelartiger, lappig aufgeschlitzter Saum aus, wie man bei den Askosporen von *Emericella variecolor* beobachten

kann. Die Askosporen unseres Pilzes vereinigen demnach zwei Merkmale verschiedener Gattungen in sich und es drängt sich die Frage an uns auf, wohin denn nun dieser merkwürdige Pilz zu stellen ist. Ciferri (4) hat in seiner Veröffentlichung über *Emericella variecolor* ein Schema für die *Eurotiaceae* angegeben, das zwar nicht alle dahin gehörigen Gattungen aufzählt, jedoch immerhin der Einteilung nach Clements und Shear (5) gegenüber gewisse Vorteile zu bieten scheint. In diesem Schema werden die Askusfrüchte mit „Hülle"-Zellen der Gattung *Emericella* zugewiesen, während die mit einem flügelartigen Saum versehenen Askosporen zu *Samarospora* gestellt werden. Da bei unserem Pilze die „Hülle"-Zellen fehlen, käme also *Emericella* nicht in Frage. Ebensowenig passt der Pilz in die Gattung *Samarospora* hinein wegen der leistenförmigen Vorsprünge der Askosporen. Auf Grund dieser Ueberlegungen müssen wir denn auch die Notwendigkeit zur Aufstellung einer neuen Gattung für unseren Pilz als zu recht bestehend erachten. Diese Gattung, welche wir *Emericellopsis* nennen möchten, würde am besten seinen Platz in dem Ciferri'schen Schema neben *Emericella* finden.

Emericellopsis nov. gen.

Perithezien kugelig, braunschwarz, mit einer aus mehreren Schichten dichtverflochtener Hyphen bestehender Wand bekleidet, mit scheitelständigem Porus sich öffnend. Asci kugelig, hyalin, 8 Sporen enthaltend. Askosporen ellipsoidisch, leicht braun gefärbt, mit einem grossen Oeltropfen und 2—5 dunklen, leistenförmigen, sich an den Polen vereinigenden oder sich nähernden Vorsprüngen, von denen je ein hyaliner, sternförmig oder lappig aufgeschlitzter Saum ausgeht. Diese Säume sind bei Vorderansicht der Sporen als peitschenförmiger Ausläufer sichtbar. Als Konidienform tritt ein Cephalosporium auf.

Lateinische Beschreibung:

Peritheziis globosis brunneonigris, peridio circumdatis hypharum dense intertextarum, poro aperiente. Ascis globosis hyalinis, octosporis. Ascosporis ellipsoideis subbrunneis cum magna gutta oleagina et 2—5 zonis, inter utrosque polos exstantibus, quae omnes praeditae sunt hyalina margine gelatinosa cum digitiformibus processibus. Forma conidiophorae Cephalosporium est.

Die Beschreibung des Pilzes lautet:

Emericellopsis terricola nov. spec.

Perithezien kugelig, braunschwarz, mit einer aus mehreren Schichten dichtverflochtener Hyphen bestehender, etwa 10 μ dicker Wand, bei der Reife mit scheitelständigem Porus sich öffnend, meist 80—90 μ gross.

Asci kugelig, mit hyaliner Wand, vergänglich, 8 Sporen enthaltend, 14—16 μ gross.

Askosporen ellipsoidisch, leicht braun gefärbt, (8—8,7) × (4,7—5,3) μ (Mittel aus 50 Sporen 8,49 × 5,12 μ), mit einem 4 μ grossen, hyalinen Oeltropfen und 2—5 dunklen, leistenförmigen, sich an den Polen vereinigenden oder sich daselbst nähernden Vorsprüngen, von denen je ein hyaliner, sternförmig oder lappig aufgeschlitzter Saum ausgeht, welcher bei Vorderansicht der Sporen als peitschenförmiger Ausläufer erscheint.

Cephalosporium-Form.

Konidienköpfchen etwa 5—7 μ gross.

Konidienträger lang und schmal, gerade, meist in der Mitte etwas erweitert, 30—40 μ lang, an der Basis 2—3 μ dick, hyalin.

Konidien ellipsoidisch oder eiförmig, hyalin, oft mit einem oder zwei Oeltröpfchen, (6—8) × (3,3—4) μ (Mittel aus 50 Konidien 5,82 × 3,45 μ), meist (5,3—6,7) × (3—4) μ.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: kleine rosafarbene Kolonien mit glatter, feuchter Haut. Unterseite rötlich gelb. Desgl. nach 24 Tagen: Kolonien 4 cm, bestehend aus einer feuchten, rosafarbenen Haut, Farbe zwischen 91 und 116 (Code des Couleurs), mit zahlreichen radiären Falten. Keine Perithezien. Unterseite rötlich gelb.

Auf Haferflocken-Agar in einer Petrischale nach 2 Monaten: Kolonien 5—6 cm, bestehend aus zahlreichen Perithezien, welche in schönen Zonen angeordnet sind (8 Zonen auf 1 cm). An den Stellen, wo die Perithezien entstanden sind, ist der Agar durchsichtig geworden.

Hab. Aus der Erde in Baarn (Frl. N. Schierbeek).

Lateinische Beschreibung:

Peritheziis globosis brunneonigris peridio 10 μ crasso, 80—90 μ. Ascis globosis octosporis, 14—16 μ. Ascosporis ellipsoideis subbrunneis (8—8,7) × (4,7—5,3) μ cum magna hyalina gutta oleagina et 2—5 zonis exstantibus inter utrosque polos quae omnes praeditae sunt hyalina margine gelatinosa cum digitiformibus processibus.

Forma cephalosporii: Caespitulis 5—7 μ. Conidiophoris longis et tenuibus, rectis, in medio largioribus, 30—40 μ longis, basi 2—3 μ largis, hyalinis. Conidiis ellipsoideis vel ovoideis hyalinis saepe biguttatis, plerumque (5,3—6,7) × (3—4) μ.

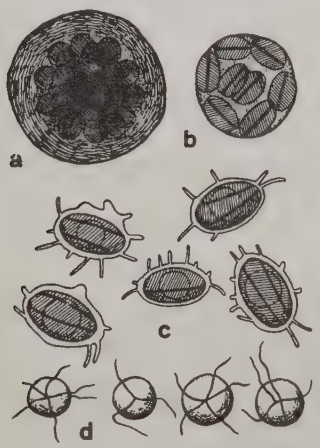

Fig. 1. *Emericellopsis terricola.*

a. Perithezium. Vergr. 245 ×.
b. Askus. Vergr. 750 ×.
c. Askosporen. Vergr. 1080× (Oelimmersion).
d. Askosporen. Vorderansicht. Vergr. 1080 × (Oelimmersion).

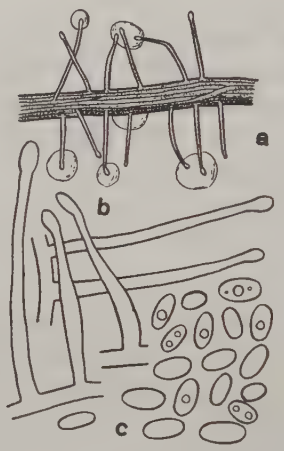

Fig. 2. *Emericellopsis terricola.* (Cephalosporium-Form).

a. Hyphenstrang mit Konidienträgern. Vergr. 245 ×.
b. Konidienträger. Vergr. 750 ×.
c. Konidien. Vergr. 750 ×.

Die Varietät unterscheidet sich von der Hauptform nur dadurch, dass die Säume der Askosporen glatt sind statt eingeschlitzt. Wir nennen diese Varietät:

Emericellopsis terricola var. *glabra* nov. var.

Auch das Wachstum auf Bierwürze-Agar in Petrischalen ist verschieden, wie aus Folgendem hervorgeht:

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 24 Tagen: Kolonie 4 cm, im Zentrum stark gefaltet und etwas erhaben; von hieraus gehen zahlreiche tiefe, radiäre Falten. Die Oberfläche ist filzig

und gelblich weiss. Die Kolonie ist am Rande etwas in den Agar hinein gewachsen. Unterseite 136. Keine Perithezien.

Desgl. nach 2 Monaten: Die Perithezien erscheinen in den zentralen Partien. Die Decke färbt sich hier etwas violett, 0471. Es werden grosse farblose oder gelbliche Wassertropfen ausgeschieden.

Auf Haferflocken-Agar ist das Wachstum der Hauptform ähnlich. Auch hier werden die Stellen, wo die Perithezien sich in schönen Zonen bilden, durchsichtig.

Lateinische Beschreibung:

A typo differt cum gelatinosa margo non digitiformibus processibus praedita, levis sit.

Penicillium (Carpenteles) euglaucum nov. spec.

Dieses *Penicillium* wurde von Dr. Marie Ledeboer in Natal (S. Afrika) isoliert von der Gerbsäure Akazie *Acacia mollesjuna* (black wattle). Es trat in Verfärbungen auf der Rinde des Baumes auf.

Der Pilz ist dadurch interessant, dass er *Carpenteles asperum* Shear nahesteht, jedoch deutliche Unterschiede mit diesem zeigt. Shear (10) glaubte mit der Beschreibung des *Carpenteles asperum* das *Penicillium glaucum* Brefeld's wiedergefunden zu haben. Die Anordnung der Asci in den Perithezien war ihm zwar nicht deutlich, doch glaubte er eine kettenförmige Entwicklung vermuten zu können. Emmons (6) wies jedoch nach, dass bei *Carpenteles asperum* die Asci seitlich an den askogonen Hyphen entstehen und nicht in Ketten. Wir haben nun leicht feststellen können, dass bei *Penicillium euglaucum* die Asci wohl in Ketten entstehen. Damit würde dieses *Penicillium* der Beschreibung Brefeld's besser genügen wie *Carpenteles asperum* und auch wie *Penicillium egyptiacum* van Beyma (1), dass von Emmons ebenfalls hierher gezogen wird.

Die Entwicklung der Konidienträger steht bei unserem *Penicillium* erheblich hinter derjenigen der Perithezien zurück. Auf fast allen Nährböden ist die Bildung der Konidien spärlich. Die Konidienträger sind zart, glatt, 2—3 μ breit. Oft sind sie einfach oder gabelig verzweigt, ohne Metulae. Bei üppiger Entwicklung jedoch endigen die Träger in 3 Aestchen, deren Metulae oben blasig erweitert sind. Die Grösse dieser Metulae ist 10—12 μ, die Breite an der Basis 3 μ, am Scheitel 3,7—4,7 μ. Die Sterigmen sind flaschenförmig, schmal, oft mit deutlichem Halse, 3—6 an der Zahl. Die Konidien sind kugelig

(Unterschied mit *Carpenteles asperum*, wo sie länglich rund sind) und mit Oelimmersion etwas rauh. Die Grösse beträgt 2—2,3 μ (— 2,7 μ); sie entstehen in langen divergierenden Ketten, mit deutlichen Disjunktoren. Dem Habitus der Konidienträger nach könnte der Pilz in die Gruppe Biverticillata-symmetrica nach Thom eingereiht werden.

Die Perithezien entwickeln sich auf allen Nährböden in grossen Massen. Sie sind anfangs farblos, später, besonders in Massen, zeigen sie eine hell-graubraune Farbe. Die Perithezienwand besteht aus dickwandigen, polygonalen Zellen und zeigt eine derartige Härte, dass die Perithezien kaum von einem Deckgläschen zerquetscht werden können. Sie sind von einem zarten, weissen Myzel überwachsen, worin zahlreiche farblose Wassertröpfchen. Die Grösse der Perithezien beträgt ungefähr 300—400 μ. Die Asci entstehen in Ketten und sind (8—9) × (6,7—7,3) μ gross. Sie enthalten 8 Sporen. Die Askosporen sind kurz ellipsoidisch, meist (3,3—4) × (3—3,3) μ gross (Unterschied mit *Carpenteles asperum*, wo dieselben (2,8—3) × (2—2,3) μ gross sind). Mit Oelimmersion betrachtet zeigt es sich, dass sie etwas stachelig sind. Ebenso wie die Askosporen von *Carpenteles asperum* besitzen sie eine deutliche, äquatorial verlaufende Leiste. Diese ist jedoch mehr hervorragend wie bei *Carpenteles asperum*, eine Furche zwischen den Randleisten wurde nicht beobachtet.

Die Beschreibung des Pilzes lautet wie folgt:

Penicillium (*Carpenteles*) *euglaucum* nov. spec.

Konidienträger meist als kurze Aeste fertiler Hyphen, 2,3—2,7 μ breit, glatt, einfach oder gabelig verzweigt, bei üppiger Entwicklung in zumeist 3 Aestchen endigend.

Metulae keulig, glatt, 10—12 μ lang, an der Basis 3 μ breit, am Scheitel zu einer 3,7—4,7 μ breiten Blase erweitert.

Sterigmen flaschenförmig, lang und schmal mit deutlichem Halse, 3—6 an der Zahl.

Konidien in langen, divergierenden Ketten mit deutlichem Disjunktor, klein, kugelig, mit Oelimmersion betrachtet rauh, 2—2,3 μ (— 2,7 μ).

Perithezien massenhaft, kugelig oder länglich rund, steinhart, anfangs farblos, später, besonders in Massen, hell-graubraun, mit harter Wand aus dickwandigen, polygonalen Zellen, von einem spärlichen, weissen Myzel überwachsen, 300—400 μ gross.

Asci kugelig oder länglich rund, in Ketten entstehend, (8—9) × (6,7—7,3) μ gross, 8 Sporen enthaltend.

Askosporen ellipsoidisch, mit Oelimmersion betrachtet etwas rauh oder stachelig, mit einer deutlichen, äquatorial verlaufenden Leiste, (3,3—4) × (3 —3,3) µ, hyalin.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: Langsam sich ausbreitende Kolonien, filzig-wollig, weiss, 2,5 cm im Durchmesser, die grösseren mit 4—5 tiefen, radiären Falten und mehreren deutlichen Zonen. Rand feinwollig. Farbloser Saum 0,5 mm. Die Kolonien sind am Rande etwas in den Agar hinein gewachsen. Kein Geruch. Noch keine Perithezien. Unterseite mit Falten und Zonen, im Zentrum der Kolonien etwas hohl, rötlich gelb. Spärliche Konidienbildung.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: stark gewölbte Haut mit vielen weissen Perithezien und zahlreichen farblosen Wassertröpfchen. Oben im Röhrchen beginnende Konidienbildung, Farbe zwischen 373 und 367. Rückseite 158, stellenweise etwa 78.

Auf Kirsch-Agar: flache Haut mit zahlreichen Perithezien, unten im Röhrchen in Zonen. Zahlreiche Wassertropfen. Oben beginnende Konidienbildung. Rückseite intensiv rot, etwa 43—44.

Auf Möhre: ganz bewachsen mit einer faltigen Haut, worauf zahlreiche Perithezien, weiss mit bräunlichem Stiche. Grosse farblose Wassertropfen. Das Stück ungefärbt.

Auf Kartoffelstück: fast ganz bewachsen mit einer faltigen Haut, worauf zahlreiche Perithezien, weiss bis hell-bräunlich, stellenweise ergrünt durch Konidienbildung, 343.

Auf Raulin: stark gefaltete weisse Haut. Keine Perithezien oder Konidien.

Auf Reis: 1 cm tief gewachsen, oben filziges, weisses Myzel. Der Reis fast farblos.

Auf Haferflocken- oder Maismehl-Agar: zahlreiche Perithezien, in Massen hell-graubraun. Luftmyzel fehlt fast ganz. Keine Konidien. Zahlreiche farblose Wassertropfen.

Hab. Von Verfärbungen der Rinde einer Akazie („Wattle") in Natal (M. Ledeboer).

Lateinische Beschreibung:

Conidiophoris 2,3—2,7 µ levibus, simplicibus vel uno ramo praedites. Metulis claviformibus, levibus, 10—12 µ longis, 3 µ largis, apice largioribus usque ad 3,7—4,7 µ; 3—6 sterigmis lagoeniformibus longis et an-

gustis. Conidiis longe catenatis cum disjunctore, globosis 2—2,3 μ.
Peritheziis numerosis globosis vel oblonge globosis plus vel minus brunneis 300—400 μ. Ascis globosis vel oblonge globosis, catenatis, octosporis, (8—9) × (6,7—7,3) μ. Ascosporis ellipsoideis paulum rudibus aequatoriali zona (3,3—4) × (3—3,3) μ praeditis.

Fig. 3. *Penicillium euglaucum.*
Konidienträger und Konidien. Vergr. 750 ×.

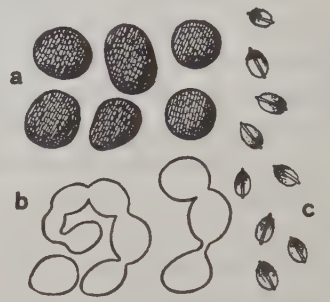

Fig. 4. *Penicillium euglaucum.*
a. Perithezien. Vergr. 30 ×.
b. Asci. Vergr. 750 ×.
c. Askosporen. Vergr. 1080 × (Oelimmersion).

Penicillium (Carpenteles) baarnense nov. spec.

Aus der Erde in Baarn isolierten wir ein *Penicillium*, welches auf kohlehydrathaltigen Nährböden zahlreiche Perithezien erzeugte. Ein Vergleich mit den in der Sammlung des Centraalbureau vorhandenen perithezienführenden Arten überzeugte uns davon, dass wir hier mit einer unbekannten Art zu tun hatten. Dieses *Penicillium* wächst auf verschiedenen Nährböden wie Bierwürze-Agar, Kartoffelstücken, u.a.m. fast genau so wie *Carpenteles asperum*, während die reifen Perithezien in der Farbe und Gestalt denen von *Penicillium egyptiacum* ähneln.

Auf den meisten Agar-Nährböden bildet es filzige, weisse Kolonien, worin die Perithezien etwa vom zehnten Tage an anfangen zu erscheinen, unter gleichzeitiger Bildung grosser, farbloser Wassertropfen, während dagegen die Konidienerzeugung fast ganz unterbleibt.

Die Konidienträger sind entweder einfach oder weisen einen Seitenast auf, der in gleicher Höhe endigt. Am Scheitel sind sie oft erweitert bis zu 4 μ, mit etwa 4—6 Sterigmen. Letztere sind flaschenförmig und schnüren die ovalen bis subglobosen Konidien in langen parallelen oder etwas divergierenden Ketten ab. Nach dem Habitus der Konidienträger gehört der Pilz in die Gruppe Monoverticillata ramigena nach Thom. Es ist jedoch sehr wohl möglich, dass beim Weiterzüchten die Konidienträger auf die Dauer eine stärkere Verzweigung aufweisen werden,

wie das auch bei *Penicillium egyptiacum* der Fall gewesen ist.

Die Perithezien entstehen besonders auf Maismehl- und Haferflocken-Agar in grossen Massen. Anfangs von weissem, filzigem Myzel bedeckt, liegen sie in älteren Kulturen dem Nährboden frei auf; sie sind kugelig, glatt und leicht gelbbraun gefärbt. Die kugeligen Asci, welche kettenförmige Anordnung zeigen, enthalten 6—8 Sporen. Letztere sind oval, stark lichtbrechend und von einer deutlich vorspringenden Leiste umgeben. Durch die kettenförmige Anordnung der Asci unterscheidet der Pilz sich von *Penicillium luteum, Ehrlichii, javanicum, Brefeldianum* und *asperum*. Ihm am nächsten kommen auf Grund der Askosporen *Penicillium stipitatum* und *Penicillium egyptiacum*. Bei *P. stipitatum* jedoch weisen die Askosporen nur eine ganz dünne vorspringende Leiste auf, ausserdem sind sie bedeutend kleiner. Bei *P. egyptiacum* sind die Askosporen nicht nur kleiner, sondern auch anders gestaltet, nämlich an den Polen etwas abgeflacht, während eine deutlich sichtbare Leiste fehlt.

Wir betrachten deshalb diesen Pilz als neu und lassen die Beschreibung unten folgen.

Penicillium (Carpenteles) baarnense nov. spec.

Rasen flach, filzig, weiss, erst spät teilweise ergrünend durch Konidienbildung. Die Bildung der Perithezien geht mit der Erzeugung zahlreicher farbloser Wassertropfen einher.

Konidienträger nicht zahlreich, einfach oder mit einem Seitenast, 3—3,3 μ dick, am Scheitel meist erweitert bis zu 4 μ, glatt, verhältnismässig kurz, etwa 30—60 μ lang, mit 4—6 flaschenförmigen Sterigmen.

Konidien oval bis subglobos, hyalin, glatt, in langen parallelen oder etwas divergierenden Ketten, meist mit Disjunktoren versehen, (3,3—4) × (2,7 — 3,3) μ.

Perithezien gesellig, kugelig, glatt, mit lederartiger Wand aus dickwandigen, polygonalen Zellen, leicht gelbbraun gefärbt, 200—300 μ gross. Ein Ostiolum konnte nicht beobachtet werden, die reifen Perithezien platzen an einer Stelle auf und entledigen sich so der Sporen.

Asci kugelig, 6—8 Sporen enthaltend, bei der Entwicklung kettenförmig angeordnet, 10—12 μ gross.

Askosporen oval, stark lichtbrechend, glatt, mit einer deutlich vorspringenden, etwa 0,92 μ breiten Leiste versehen, (4,7—5,3) × (4—4,7) μ.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 14 Tagen: Kolonie 6,5 cm, flach filzig, weiss, mit einzelnen untiefen, radiären Falten. Einzelne Segmente sind ein wenig braungelb gefärbt wegen beginnender Perithezien-Bildung, mit grossen Wassertropfen daselbst. Rand flach, faserig. Farbloser Saum 1—1,5 mm. Kaum Geruch. Unterseite etwa 0271.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar und Kirsch-Agar eine filzige, unebene Decke, 303 B—303 C, mit zahlreichen jungen Perithezien.

Auf Möhre: das Stück ganz bewachsen mit einer weissen, gefalteten Haut. Zahlreiche Wassertröpfchen, spärliche Perithezien-Entwicklung.

Auf Kartoffelstück: das Stück ganz bewachsen mit einer weissen, gefalteten Haut, unten im Röhrchen glatt und feucht. Spärliche Entwicklung von Konidien und Perithezien.

Auf Haferflocken- und Maismehl-Agar: flache, filzige, weisse Decken mit zahlreichen Perithezien und vielen farblosen Wassertröpfchen.

Hab. Aus der Erde in Baarn.

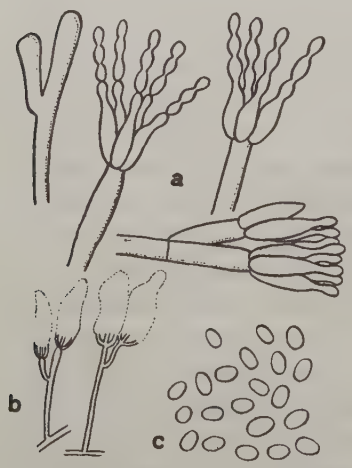

Fig. 5. *Penicillium baarnense.*

a. Konidienträger. Vergr. 750 ×.
b. Desgl. Vergr. 245 ×.
c. Konidien. Vergr. 750 ×.

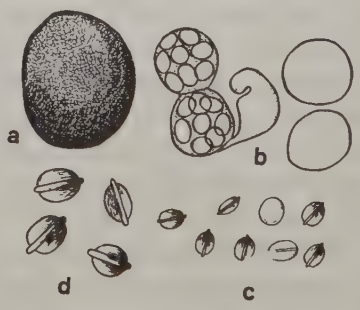

Fig. 6. *Penicillium baarnense.*

a. Perithezium. Vergr. 80 ×.
b. Asci. Vergr. 750 ×.
c. Askosporen. Vergr. 750 ×.
d. Desgl. Vergr. 1080 × (Oelimmersion).

Lateinische Beschreibung:

Peritheziis numerosis globosis levibus, coriaceo pariete, subflaveobrun-

neis, 200—300 μ. Ascis globosis, 6—8 sporis continentibus, 10—12 μ. Ascosporis ovoideis levibus manifeste eminenti zona circumdatis. Conidiophoris simplicibus vel uno ramo praeditis, 3—3,8 μ crassis apice plerumque crassioribus, usque ad 4 μ, 4—6 lagoeniformibus sterigmis. Conidiis ovoideis vel subglobosis hyalinis, levibus longis parallelibus vel paulim divergentibus catenis cum disjunctore, (3,3—4) × (2,7—3,3) μ.

Penicillium novae-zeelandiae nov. spec.

Dieses *Penicillium* wurde von J. C. Neill in Palmerston North (Neu-Seeland) aus einem Apothezium einer Sclerotinia isoliert und dem C.B.S. als No. 684 zugeschickt. Herr Neill war so liebenswürdig uns, nachdem der Pilz als eine neue Art erkannt worden war, die Beschreibung zu überlassen, worauf wir dieses *Penicillium* dem Lande der Herkunft gewidmet haben.

Was am meisten bei diesem *Penicillium* auffällt sind die schwarzen Sklerotien, welche in grossen Massen gebildet werden. Sie treten in längeren, mehrfach verzweigten Verbänden auf, wie dies auch der Fall ist bei den *Botrytis*-Arten der Zwiebelpflanzen, und bilden auf künstlichen Nährböden in Petrischalen schöne Zonen. Die Gestalt der einzelnen Sklerotien, welche 0,5—0,75 mm gross sind, ist im allgemeinen eine elliptische. Die Wand derselben ist lederartig und besteht aus dunkelbraunen, rundlichen bis polygonalen Zellen. Auf den meisten Nährböden werden die Sklerotien bald von einem hochwolligen, weissen, schnellwüchsigen Myzel überdeckt, dessen fertile Hyphen die Konidienträger hervorbringen. Diese Konidienträger sind sehr oft unverzweigt und erreichen eine grosse Länge, bis zu 500 μ und darüber; anderenfalls besitzen sie einen Seitenzweig. Die Aussenwand der Konidienträger ist, ebenso wie diejenige der Hyphen, durch zahlreiche darauf niedergeschlagenen Körnchen granuliert. Die 3—4 μ breiten Hyphen bilden mehr oder weniger dicke Stränge, welche, besonders am Rande grösserer Kolonien, einen mehrere mm hohen, faserig-wolligen, weissen Wall bilden.

Die Konidien sind 2,3—2,7 μ gross, kugelig, glatt. Sie werden in langen, geschlängelten Ketten abgeschnürt. Was die systematische Stellung anbetrifft, gehört der Pilz in die Gruppe Asymmetrica-Funiculosa nach Thom.

Da der Pilz sich erheblich von den anderen bekannten, sklerotienführenden Penicillien unterscheidet, wurde er als neue Art beschrieben. Diese Beschreibung lautet folgendermaassen:

Penicillium novae-zeelandiae nov. spec.

Rasen bestehend aus zahlreichen schwarzen Sklerotien, von einem wolligen, weissen Myzel umgrenzt.

Konidienträger meist unverzweigt, seltener mit einem Seitenast, bis zu 500 μ lang und darüber, 3—3,3 μ dick, allseitig durch darauf niedergeschlagene Körnchen granuliert.

Fig. 7. *Penicillium novae-zeelandiae.*

a. Konidienträger. Vergr. 750 ×.
b. Konidienträger. Vergr. 245 ×.
c. Konidien. Vergr. 750 ×.
d. Sklerotien. Vergr. 30 ×.

Metulae keulenförmig, 8—10 μ lang und 3—4 μ dick, meist ebenfalls körnig, 3—5 an der Zahl.

Sterigmen flaschenförmig, mit kurzem Halse, meist 10 μ lang und 2,5—2,7 μ dick, 2—4 an der Zahl, glatt, dicht beisammen stehend.

Konidien zahlreich, kugelig, glatt, hyalin, 2,3—2,7 μ, in langen geschlängelten, oft divergierenden Ketten mit deutlichem Disjunktor.

Sklerotien massenhaft, in längeren, mehrfach verzweigten Verbänden auftretend, von sehr verschiedener Gestalt, jedoch die ellipsoidische vorherrschend, schwarz, meist von einem ganz dünnen, weissen Myzel überwachsen, oft der Pilzhaut etwas eingesenkt, (400—800) × (300—500) μ gross, von einer aus dickwandigen, polygonalen Zellen bestehenden, lederartigen Wand umgeben.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 20 Tagen: grosse Kolonien, etwa 4 cm im Durchmesser, bestehend aus zahlreichen, in schönen Zonen angeordneten, schwarzen Sklerotien, welche von einem dünnen, weissen Myzel überwachsen sind. Die Kolonien sind je von einem dicken Wulst aus weissem, wolligem Myzel, 2—3 mm hoch, umgeben. Kaum Geruch.

Auf Röhrchen nach 7 Tagen:

Auf Bierwürze-Agar: zahlreiche Sklerotien, von einem weissen, wolligen Myzel überwachsen.

Auf Kirsch-Agar: wie vorige, die Sklerotien jedoch weniger zahlreich.

Auf Möhre: das Stück ganz bewachsen von einer faltigen Haut mit zahlreichen Sklerotien.

Auf Kartoffelstück: das Stück ganz bewachsen von einem weissen, wolligen Myzel, an der Glaswand etwas gelblich. Verhältnismässig wenig Sklerotien. Rand am Glase orangefarben, 161—156.

Auf Raulin: Kleine Kolonie, filzig-wollig, ohne Sklerotien. Rückseite 246.

Auf Haferflocken-Agar: dünnes, wolliges, weisses Myzel, fast ohne Sklerotien-Bildung.

Auf Reis: 1 cm tief gewachsen, oben weisses, wolliges Myzel ohne Sklerotien. Der Reis etwas gelblich.

Hab. Isoliert aus dem Apothezium einer Sclerotinia (J. Neill, Palmerston North, Neu-Seeland).

Lateinische Beschreibung:

Conidiophoris raro ramosis usque ad 500 μ longis, 3—3,3 μ crassis, 3—5 metulis claviformibus, 8—10 μ longis, 3—4 μ crassis; 2—4 sterigmis levibus lagoeniformibus collo brevi, 10 μ longis, 2,5—2,7 μ crassis. Conidiis numerosis globosis levibus hyalinis, 2,3—2,7 μ, longis catenis saepe divergentibus, obviis disjunctoribus. Sclerotiis numerosis plerumque ellipsoideis nigris, (400—800) × (300—500) μ, coriaceo pariete praeditis.

Bisporomyces chlamydosporis nov. gen. nov. spec.

Dieser Pilz wurde von Bakhuizen van den Brink aus der Erde in Baarn isoliert. Er wächst mit grauem bis schwarzem Myzel, von dem sich zahlreiche Phialiden erheben, mitunter unter Bildung aufstehender Hyphenbündel, von welchen dann nach allen Seiten die Phialiden abgehen. Der Pilz ist besonders dadurch interessant, weil er die Konidien genau so erzeugt, wie wir das für die frühere Gattung *Cadophora*, jetzt *Phialophora*, festgestellt haben (2). Bei unserem Pilze wird jedoch nicht eine Konidie jedesmal gebildet sondern deren zwei, welche entweder zu gleicher Zeit oder kurz nach einander erscheinen. Dadurch konnte der Pilz der Gattung *Phialophora* nicht zugeteilt werden, sodass wir ihn als Typus einer neuen Gattung, die wir *Bisporomyces* nennen möchten, angenommen haben.

Die Hyphen sowohl wie die Phialiden sind bei diesem Organismus braun gefärbt. Nur am Scheitel sind letztere etwas heller oder fast farblos. Die Phialiden stehen meist dicht beisammen, sie sind sehr verschiedener Grösse, meist 80—100 μ, können jedoch eine Länge

von etwa 180 μ erreichen. Dies kommt daher, dass manche Phialiden am Scheitel nicht zur Bildung von Konidien gelangen; die aus dem Innern hervorgestülpte hyaline Membran nimmt in dem Falle fortwährend an Länge zu, färbt sich dabei allmählig braun und erzeugt nun nach der Reife die Konidien (Fig. 8*a* rechts). Dergleiche Phialiden sind daran zu erkennen, dass sie etwa in halber Höhe eine Verbreiterung aufweisen (Fig. 8*d*). Wie schon erwähnt werden die Konidien nach Art der Gattung *Phialophora* erzeugt, das heisst, dass dieselben ihren Ursprung einer inneren Membran entnehmen. In den meisten Fällen entstehen zwei Konidien zu gleicher Zeit oder jedenfalls kurz nach einander; die erstentstandene Konidie erscheint seitlich am Scheitel. Wie bei *Phialophora* kommt es auch hier vor, dass eine bereits abgestossene Konidie noch einige Zeit durch die hyaline Membran mit der Phialide verbunden bleibt.

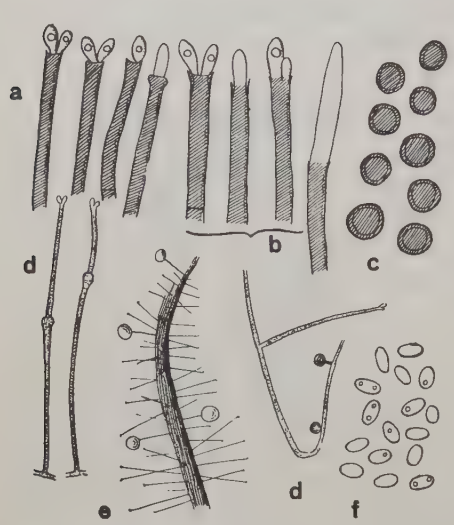

Fig. 8. *Bisporomyces chlamydosporis.*

a. Phialiden. Vergr. 750 ×.
b. Phialiden. Vergr. 1080 × (Oelimmersion).
c. Chlamydosporen. Vergr. 750 ×.
d. Durchwachsene Phialiden und Chlamydosporen und Phialiden an demselben Faden. Vergr. 245 ×.
e. Spitze eines Hyphenbündels. Vergr. 80 ×.
f. Konidien. Vergr. 750 ×.

Die Konidien sind hyalin oder leicht gefärbt und oft mit einem oder zwei Oeltröpfchen versehen. Ihre Gestalt ist mehr oder weniger eiförmig. Neben Konidien erzeugt der Pilz noch zahlreiche Chlamydosporen. Diese kommen besonders in älteren Kulturen vor, und zwar sowohl sitzend am Myzel wie auf kleinen Stielchen. Sie sind kugelig, braun und haben eine doppelte Wand.

Für die Gattung stellen wir folgende Diagnose auf:

Bisporomyces nov. gen.

Rasen rauchgrau bis schwärzlich. Hyphen braun, septiert. Phialiden lang und schmal, braun, septiert, am Scheitel zwei Konidien zugleich erzeugend nach Art der Gattung *Phialophora*. Konidien zahlreich, hyalin bis leicht gefärbt.

Lateinische Beschreibung:

Hyphis brunneis septatis, phialidibus longis et tenuibus brunneis septatis, cacumine bina conidia substringentibus, modo Phialophorae. Conidiis numerosis hyalinis vel leviter coloratis.

Die Beschreibung des Pilzes lautet folgendermaassen:

Bisporomyces chlamydosporis nov. spec.

Rasen flach filzig, oft mit Erhöhungen und Falten, grau bis schwarz, langsam wachsend, an der Impfstelle oft mit Hyphenbündeln.

Phialiden lang und schmal, septiert, braun, von sehr verschiedener Länge, etwa 60—180 μ lang und 2,7—3 μ breit, am Scheitel zwei Konidien zu gleicher Zeit nach Art der Gattung *Phialophora* erzeugend.

Konidien mehr oder weniger eiförmig, hyalin bis leicht gefärbt, oft mit einem oder zwei Oeltröpfchen, (3,7—4,7) × (2 — 3) μ, meist (4,3—4,7) × 2,7 μ, zahlreich, in feuchter Umgebung sich zu falschen Köpfchen zusammenballend.

Chlamydosporen kugelig, braun, mit doppelter Wand, 5—6 μ im Durchmesser, besonders in älteren Kulturen zahlreich, an den Hyphen sitzend oder auf kleinen Stielchen.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 25 Tagen: Kolonie 6 cm, rauchgrau, filzig, im Zentrum mit Erhöhungen und Falten, sowie Hyphenbündeln. Schwacher Geruch. Unterseite schwärzlich.

Auf Röhrchen nach 10 Tagen:

Auf Haferflocken- und Maismehl-Agar: Flache, schwarze, submerse Haut. Kein Luftmyzel.

Auf Kartoffelstück und Möhre: Fast ganz bewachsen mit einer schwarzen, feuchten, glatten Haut, ohne Luftmyzel.

Hab. Aus der Erde in Baarn (Bakhuizen van den Brink).

Lateinische Beschreibung:

Phialidibus longis et tenuibus septatis brunneis, (60—180) × (2,7—3) μ, apice bina conidia substringentibus, Phialophorae instar.

Conidiis ovoideis hyalinis vel leviter coloratis, plerumque 1—2 oleaginis guttis, plerumque (4,3—4,7) × 2,7 μ, caespitulatis. Chlamydosporis numerosis brunneis, 5—6 μ sedentibus vel breviter pedicellatis.

Phialophora aurantiaca nov. spec.

Dieser Pilz wurde dem C.B.S. unter No. B 45 zugeschickt von

T. Tomlinson, Minworth (Engl.), der ihn im Water Pollution Research Laboratory aus Abwässern isolierte.

Er wächst anfangs ungefähr so wie *Ph. lignicola* (Nannf.) G. Goid., isoliert aus Holzschliff und steht diesem Pilze gewiss auch am nächsten. Während jedoch *Ph. lignicola* bald seine blass orangegelbe Farbe in grauschwarz bis schwarz ändert, behält unser Pilz seine Farbe bei, die er meist noch vertieft zu lebhaft rotorange. Die Entwicklung der Phialiden zeigt ebenfalls Aehnlichkeit mit *Ph. lignicola*, indem diese oft aus kugelig aufgetriebenen Seitenästen der Hyphen hervorgehen. Im allgemeinen stehen die Phialiden den mit Oeltröpfchen gefüllten Hyphen entlang, einzeln oder in kleinen Gruppen, oder als dichte Rosetten auf kurzen Stielen, oft zwei übereinander. Die Konidien, welche einer inneren Membran entstammen, werden in grossen Massen erzeugt. Sie sind eiförmig oder ellipsoidisch, mit zwei Oeltröpfchen, (3—4) × (2—3) μ gross; es gibt aber auch grössere, (6—7) × (3—4) μ. Einzeln hyalin besitzen sie in Massen eine gelbliche Farbe. Da der Pilz zweifellos eine unbekannte Art darstellt, wird er unten als *Phialophora aurantiaca* n. sp. beschrieben.

Ehe wir zur Beschreibung schreiten, möchten wir noch Folgendes bemerken. Schon früher (2) betonten wir die Schwierigkeit, bei gewissen Arten die Bildung der Konidien aus Phialiden nachzuweisen. Dadurch war es z.B. möglich, dass Melin und Nannfeldt eine neue Gattung aufstellten (*Lecythophora*) für einen Pilz, der nach unseren Untersuchungen zur Gattung *Cadophora* (jetzt *Phialophora*) gehörte. So leicht nun diese Bildung aus Phialiden und damit das Auftreten des typischen Schnabels derselben bei einigen Arten nachzuweisen ist, so schwierig, ja unmöglich kann dies in anderen Fällen sein. Es ist nun interessant darauf hinzuweisen, dass in letzterem Falle der Pilz nicht bei *Phialophora* unterzubringen ist, sondern bei *Margarinomyces*. Schon bei dem Studium einiger zu dieser Gattung gehöriger Arten (3) war mir die Uebereinstimmung im Habitus und in morphologischer Beziehung der beiden Gattungen *Phialophora* und *Margarinomyces* aufgefallen. Beiden Gattungen gemeinsam ist oft die glatte, lebhaft gefärbte Pilzhaut, die massenhaften kleinen, oft gebogenen Konidien, die Oeltröpfchen enthaltenden Myzelien, die büschelweise Bildung der Phialophoren bzw. Konidienträger, u.s.w. Nur gelang es mir nicht mit Sicherheit die endogene Bildung der Konidien bei *Margarinomyces* nachzuweisen. Sollte dennoch später dies mal gelingen, so sind die betreffenden Arten bei *Phialophora* unterzubringen. Für heute kann man sagen, dass die *Margarinomyces*-Arten *Phialophora*-Arten sind, bei

denen der Nachweis der Phialiden nicht gelungen ist. Letztere können hier nur vermutet werden. Das will also sagen, dass es von den Arten mit deutlichen Phialiden und dessen auffälligen „Schnäbeln" bis zu den Konidienträgern mit Sterigmen alle möglichen Uebergänge gibt, wodurch es schwierig ist, hierher gehörige Pilze richtig zu deuten.

Phialophora aurantiaca nov. spec.

Phialiden zahlreich, sehr verschiedener Gestalt, manchmal aus kugelig aufgetriebenen Seitenästen hervorgehend, sonst langgestreckt flaschenförmig, einfach oder büschelig beisammen, oder als Rosetten auf kurzen Stielen entstehend, 5—20 μ lang, an der breitesten Stelle 3—5 μ dick, oft mit Oeltröpfchen gefüllt, mit undeutlichem Schnabel.

Konidien massenhaft, einzeln hyalin, in Massen gelblich, eiförmig bis kurz ellipsoidisch, mit zwei Oeltröpfchen, (3—4) × (2—2,3) μ oder grösser, ellipsoidisch, (5—7) × (2,7—3,3) μ.

Fig. 9. *Phialophora aurantiaca.* Phialiden und Konidien. Vergr. 750 ×.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: langsam wachsend, Kolonie 3 cm, bestehend aus einer glatten Haut. Farbe zwischen 141 und 111, meist mit Falten. Rand heller bis fast farblos. Ueberwuchs von zartem, spärlichem, weissem Myzel. Kein Geruch. Unterseite der Kultur 182—161.

Desgl. nach 20 Tagen: Kolonie 4 cm, mit einzelnen, radiären Falten häutig, orangefarben, 192, mit spärlichem Ueberwuchs von flockigem, weissem Myzel, mit zarten, feinen Zonen. Rand flach, farblos. Kaum Geruch. Unterseite etwas hohl, rissig, etwa 197 oder etwas heller.

Desgl. nach 50 Tagen: Schale ganz bewachsen mit einer etwas faltigen, glatten Haut, Farbe zwischen 111 und 192. Diese Haut ist überwachsen von einer dünnen Schicht aus filzig-wolligem, weissem Myzel, dass durch die rötliche Unterlage und die massenhafte Sporenbildung ebenfalls rötlich erscheint. Die Myzelschicht zeigt zarte Zonen. Der Geruch ist schwach, etwas moderig. Unterseite der Kultur 136—182.

Hab. Aus Abwässern in Minworth, Engl. (Tomlinson).

Lateinische Beschreibung:

Phialidibus numerosis saepe e globosis cellulis nascentibus, plerumque longis lagoeniformibus, solitariis vel fasciculatis vel rosulatis, breviter pedicellatis, conidiis numerosis hyalinis biguttatis, (3—4) × (2—2,3) μ vel majoribus 5,7 × (2,7—3,3) μ.

Phialophora lutea-olivacea nov. spec.

Dieser Pilz stammt aus den Abwässern der Schleiferei Byske in Munksund (Schweden) und wurde dem C.B.S. zur Untersuchung zugesandt. Er fällt auf durch eine schön olivgelbe glatte Haut. Der Nachweis der Phialiden gelang nur schwierig, junge Kulturen machten unter dem Mikroskop den Eindruck einer *Margarinomyces*. Der uns unbekannte Pilz wurde wie folgt neu beschrieben:

Phialophora lutea-olivacea nov. spec.

Phialiden zahlreich, sehr verschiedener Gestalt und Grösse, 4—5 μ dick, meist mehr oder weniger flaschenförmig, oft büschelig beisammen, mit einigen kleinen Oeltröpfchen im Innern.

Konidien massenhaft, hyalin, ellipsoidisch oder länglich mit zwei, seltener drei oder mehr, deutlich sichtbaren Oeltröpfchen, (4,7—6,7) × (2,3—2,7) μ.

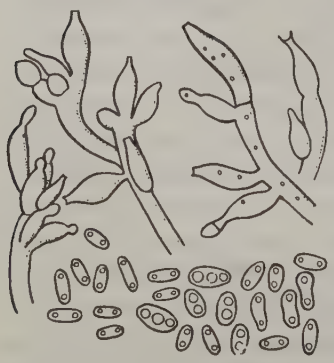

Fig. 10. *Phialophora lutea-olivacea.*
Phialiden und Konidien. Vergr. 750×.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: langsam wachsend, Kolonie etwa 3 × 2,5 cm, bestehend aus einer glatten, schön olivgelben Haut mit radiär gerichteten Falten am Rande. Im Zentrum ein etwa 1 cm hohes Büschel von weissem Myzel. Kolonie am Rande etwas in den Agar hineingewachsen. Unterseite 182 oder etwas heller.

Desgl. nach 20 Tagen: Kolonie 4 cm im Durchmesser, häutig, mit tiefen, radiären Falten, im Zentrum mit einige mm langen, weissen bis blassgelben Hyphenbündeln. Farbe der Haut im Zentrum olivgelb, nach dem Rande hin farblos. Etwa 1 cm vom Zentrum entfernt wächst eine 5 mm breite, schwarze Zone. Schwacher, etwas würziger Geruch. Unterseite der Kultur 237.

Hab. Aus Abwässern der Schleiferei Byske (Munksund, Schweden).

Lateinische Beschreibung:

Phialidibus numerosis 4—5 μ crassis, plerumque lagoeniformibus biguttatis, rarius tribus vel pluribus guttis praeditis, (4,7—6,7) × (2,3—2,7) μ.

Graphium Cartwrightii nov. spec.

Im November 1935 sandte Dr. CARTWRIGHT aus Princes Risborough (Engl.) uns diesen Pilz, der auffallend üppig wuchs auf Fussböden von Coniferenholz (u.a. *Pinus sylvestris*), auch dann, nachdem dieselben mit Kreosot behandelt worden waren. MASON, der diesen Pilz zuerst untersuchte, vermochte ihn nicht in irgend eine Gattung unterzubringen und wollte ihn mit dem vorläufigen Namen *Pachnocybe ferruginea* Berk. bezeichnen.

Der Pilz wächst auf künstlichen Nährböden, besonders auf Kirsch-Agar, anfangs nur langsam und mehr oder weniger submers, darauf entsteht ein filziges, weisses Myzel und erst nach etwa 14 Tagen erscheinen die ersten Koremien, welche bald die ganze Oberfläche bedecken. Diese Koremien sind im Mittel 600—700 μ lang, an der Basis 40—60 μ breit und bestehen aus längsverlaufenden, parallelen Hyphen, welche sich nicht nur ausschliesslich am Scheitel auflockern und ausbreiten, sondern mitunter auch an mehreren Stellen des Stieles dergleiche Spreizungen zeigen; sie sehen daher manchmal aus wie schlecht gebündelte Reiser verschiedener Länge. Die Mehrzahl derselben weist jedoch die normale Gestalt auf, mit am Ende ein unregelmässiges, rundliches Köpfchen, wo die zahlreichen Konidien abgeschnürt werden. Die Hyphen der Koremien sind braun, spärlich septiert, verzweigt, 3—6 μ breit, nach oben keulig und am Scheitel abgerundet. An diesen Hyphenenden entstehen zuerst 3—5 kleine, runde, hyaline Auswüchse, welche bald an Länge zunehmen, sich dunkel färben und alsdann die reifen Konidien darstellen, welche nach dem Ablösen noch zusammenhaften und zu grösseren Köpfchen verkleben. Bei starker Vergrösserung (Oelimmersion) konnte festgestellt werden, dass die reifen Konidien einem kleinen Stielchen aufsaszen, fast genau so, wie das bei den Basidiomyceten der Fall ist. Es will uns jedoch vorkommen, dass nicht diese Stielchen zuerst da sind, und die Konidien erst später sich daraus entwickeln, sondern die reifen Konidien scheinen nach unseren Beobachtungen an der Basis eine kleine Streckung zu erfahren, wo-

durch sie noch mit dem Mutterfaden verbunden sind, ehe sie sich von demselben loslösen.

Die Konidien sind länglich rund bis ellipsoidisch, einzellig, anfangs hyalin, später braun, (5,7—8,3) × (2,3—3,3) μ gross und sehr zahlreich. Neben dieser Graphium-Form wurde noch eine Verticillium-Form beobachtet, welche vorwiegend auf Bierwürze-Agar auftrat. Diese Form bestand aus einem grauweissen Myzel mit langen, oft nur spärlich verzweigten Trägern. Diese Träger zeigten in gewissen Abständen zwei, manchmal auch einzelne, kurze Seitenzweige, nach oben hin meist 2—3-teilige Wirtel, welche bis zu 5 länglichen, oft etwas hin und hergebogenen Sterigmen aufwiesen. Die Konidien, welche sich zu Köpfchen zusammenballen können, sind länglich, an den Enden abgerundet und meist wurstähnlich gekrümmt, hyalin. Ihre Grösse beträgt (6—7) × × (1,7—2) μ.

Bei der Bestimmung der systematischen Stellung dieses Pilzes müssen wir zuerst bemerken, dass die Gattung *Pachnocybe* (9) keine einheitliche ist, sondern zum Teil zu *Graphium* gehört, worin sie denn auch als Synonym aufgegangen ist. Weiter hat Goidànich (8) die *Stilbaceae* einer Revision unterworfen und einige neue Gattungen aufgestellt. Die von ihm behandelten Gattungen sind:

1) *Graphium* Corda (1837) em. G. Goidànich (1935)
2) *Nematographium* G. Goid. (1935)
3) *Graphiopsis* Bain. (1907)
4) *Pleurographium* G. Goid. (1935).

Für unseren Pilz scheidet *Graphiopsis* sofort aus, da bei dieser Gattung die Endteile der Stielhyphen gezähnelt sind. *Nematographium* ebenfalls, da hier die Koremien aus einfachen, unverzweigten Stielhyphen zusammengesetzt sind. Bei *Pleurographium* entstehen die Konidien pleurogen und an den Ecken der wellig gebogenen Aestchen. Wir bringen unseren Pilz deshalb zur Gattung *Graphium*, obwohl auch hier ohne Zweifel in Zukunft eine weitere Einteilung nötig sein wird. Viele Arten der Gattung *Graphium* gehören als Konidienformen zur Gattung *Ophiostoma*. Bei unserem Pilze haben wir eine höhere Fruchtform nicht aufgefunden.

Die Beschreibung des Pilzes gestaltet sich wie folgt:

Graphium Cartwrightii nov. spec.

A. Graphium-Form.

Koremien gesellig, braunrot, 600—700 μ hoch, an der Basis 40—60 μ breit.

Stiel oft durch spreizende Kopfhyphen mehrmals unterbrochen, sonst mehr oder weniger glatt, aus mehreren Hyphen zusammengesetzt, gerade.

Stielhyphen braun, 3—6 μ breit, keulenförmig, verzweigt, spärlich septiert, am rundlichen Scheitel 5 bis 7 Konidien zu gleicher Zeit erzeugend.

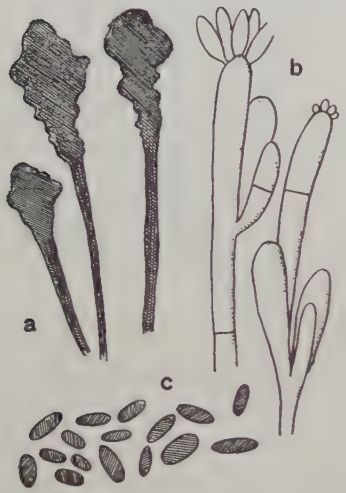

Fig. 11. *Graphium Cartwrightii.*

a. Koremien. Vergr. 80 ×.
b. Konidienträger. Vergr. 750 ×.
c. Konidien. Vergr. 750 ×.

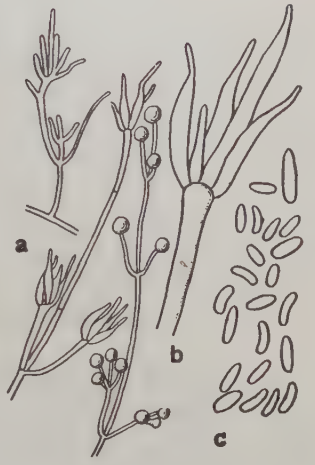

Fig. 12. *Graphium Cartwrightii.* (Verticillium-Form).

a. Konidienträger. Vergr. 245 ×.
b. Konidienträger. Vergr. 750 ×.
c. Konidien. Vergr. 750 ×.

Konidien einzellig, anfangs hyalin, später rotbraun, in Massen braunrot, (5,7—8) × (2,3—3,3) μ — meist (5,6—7,3) × (2,3—2,7) μ — sich bei der Reife wie von kleinen Stielchen abschnürend.

B. Verticillium-Form.

Rasen flach filzig bis flach wollig, weisslich, langsam wachsend, undeutliche Zonen bildend.

Konidienträger lang, fädig, 3—4 μ breit, hyalin, mit opponierten oder quirlständigen Aestchen, 2—4 an der Zahl, 10—40 μ lang und 3—4 μ breit.

Sterigmen lang und schlank, 20—30 μ lang und 3—3,3 μ breit, nach der Spitze zu sich verjüngend und meist daselbst gekrümmt oder hin und her gebogen, 3—6 an der Zahl, meist grössere und kleinere beisammen.

Konidien zahlreich, hyalin, länglich, an den Ecken abgerundet, meist würstchenartig gekrümmt, einzellig, (5—7,3) × (1,7—2) μ, meist (6—7,3) × 1,7 μ, durch Schleim zu 10 μ grossen Köpfchen verklebt.

Hab. Von Koniferenholz, das mit Kreosot behandelt worden war, in England (CARTWRIGHT).

Lateinische Beschreibung:

Coremiis gregariis brunneorubris 600—700 μ longis, basi 40—60 μ crassis, stipitibus ex hyphis constantibus brunneis, 3—6 μ largis, claviformibus ramosis, raro septatis, rotundo cacumine e quo quina vel septena conidia oriuntur. Conidiis primum hyalinis, deinde brunneorubris plerumque (5,7—7,3) × (2,3—2,7) μ. Forma verticillii: Conidiophoris longis, 3—4 μ crassis, 2—4 opponentibus vel coroniformibus ramis, (10—40) × 3 μ. Sterigmis longis et gracilibus, (20—30) × (3—3,3) μ, ad apicem magis tenuibus et ibi saepius curvatis. Conidiis numerosis hyalinis ellipsoideis, angulis curvatis, tomenti instar, plerumque (6—7,3) × 1,7 μ, in capitulis falsis 10 μ diam. dispositis.

Oospora colorans nov. spec.

Dr. JANSEN in Deventer isolierte von der Haut einer im Bäckereibetriebe arbeitenden Person einen Pilz, der sich als eine *Oospora* herausstellte. Von einer pathogenen Wirkung auf die menschliche Haut ist jedoch nichts bekannt.

Anfangs weiss bis kremfarben, nimmt der Pilz mit zunehmender Myzelbildung eine immer mehr intensive orangegelbe Farbe an. Auf Bierwürze-Agar in Schalen gezüchtet, produziert er einen auffallend schönen Farbstoff, der den Agar intensiv rot färbt. Dieser Farbstoff entsteht auch auf Kartoffel-Agar, wo er sich zu zahlreichen kleinen, kugeligen Gebilden zusammenballt, welche mit der Lupe betrachtet leicht wahrgenommen werden können. Auf dem ersten Blick machen sie den Eindruck von Perithezien oder Sklerotien. Der Farbstoff ist löslich in Alkohol.

Die 2—3 μ breiten Hyphen sind farblos und treten leicht zu Strängen zusammen, von welchen die Konidienträger nach allen Seiten abgehen. Letztere sind verhältnismässig kurz, etwa 20—30 μ lang, meist unverzweigt, unten 2,3—2,7 μ dick, nach der Spitze zu sich verjüngend und hier die Konidien in langen Ketten abschnürend. Diese werden in grossen Massen gebildet, sodass die Oberfläche der Nährböden oft mit einer puderigen Schicht bedeckt ist.

Da eine *Oospora* mit obengenannten Merkmalen uns nicht bekannt war, wurde der Pilz wie folgt als neue Art beschrieben:

Oospora colorans nov. spec.

Hyphen zart, 1,7—2 μ breit, gelblich, meist zu Strängen vereint.

Konidienträger lang und schmal, 20—30 μ lang, an der Basis 2,7 μ breit, sich nach oben allmählig verjüngend.

Konidien spindelförmig, hyalin, (4,7—5,3) × (2—2,3) μ, ohne Oeltröpfchen, in langen Ketten an den Trägern entstehend.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: Kolonie 2—3 cm, flach filzig, weiss bis kremfarben, am Rande mit flachen Hyphensträngen ausstrahlend, den Agar schön rot färbend. Rand scharf. Kaum Geruch. Unterseite intensiv rot, etwa 1—2. Nach 14 Tagen ist die Farbe mehr orange, 177—182—152. Unter der oberen weissen, filzigen Schicht ist das Myzel gelb, 241.

Fig. 13. *Oospora colorans*. Konidienträger und Konidien. Vergr. 750 ×.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: Decke flach filzig, weiss mit violettem Stiche, im Zentrum am Impfstrich entlang gelb, 266. Tiefe Querfalten. Der Agar ist intensiv rot gefärbt.

Auf Kirsch-Agar: flach-krauswolliges Myzel, weiss und gelb vermischt, 266. Agar rot gefärbt.

Auf Kartoffelstück: ganz bewachsen mit weissem, zum Teil submersem Myzel. Schlechtes Wachstum. Das Stück ist ungefärbt.

Auf Reis: 3 cm tief gewachsen, oben weisses und gelbes Myzel, 266—241. Der Reis färbt sich intensiv violettrot, 593—595.

Hab. Von der Haut einer im Bäckereibetriebe arbeitenden Person (Jansen).

Lateinische Beschreibung:

Conidiophoris longis et tenuibus 20—30 μ longis, basi 2,7 μ largis. Conidiis fusiformibus hyalinis, (4,7—5,3) × (2 — 2,3) μ, eguttulatis, longis catenis dispositis.

Spirotrichum musae nov. spec.

Dieser Pilz wurde von STAHEL von Bananenblättern in Suriname isoliert und dem Centraalbureau im Februar 1934 als *Verticillium* species zugeschickt. Nachdem SAITO in Japan die Gattung *Spirotrichum* aufstellte (1939) und dem C.B.S. den Typus, *Spirotrichum purpureum* zuschickte, wurde die Zugehörigkeit des angeblichen *Verticillium* von STAHEL zu dieser Gattung während einer gelegentlichen Betrachtung erkannt. Leider ist die Veröffentlichung SAITO's in japanischer Sprache gehalten, während eine lateinische Beschreibung fehlt, sodass die Umschreibung der Gattung *Spirotrichum* den wenigsten Mykologen zugänglich ist. Da wir nun dem Typus eine zweite Art zugesellen können, glauben wir berechtigt zu sein, eine Beschreibung der Gattung auf Grund der beiden Arten in deutscher und lateinischer Sprache hinzufügen zu dürfen.

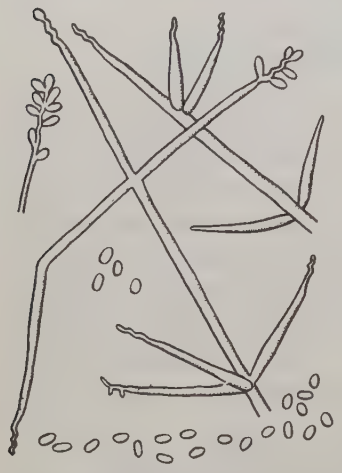

Fig. 14. *Spirotrichum musae*. Konidienträger und Konidien. Vergr. 750 ×.

Die Beschreibung der Gattung lautet wie folgt:

Spirotrichum Saito emend.

Rasen begrenzt, sammetartig, stark gefaltet. Konidienträger zart, am Scheitel wirtelig verzweigt. Wirtel höchstens aus 3—4, oft auch aus nur 2 Sterigmen bestehend, selten mehr als 2 Wirtel übereinander. Sterigmen an den Spitzen mehrfach hin und her gebogen (zackig), die Konidien an den Zacken abschnürend. Konidien klein, hyalin, zahlreich, rundlich bis eiförmig oder ellipsoidisch.

Lateinische Beschreibung:

Caespitulis terminatis numerosis rugis. Conidiophoris ramis in coronis dispositis praeditis, coronulis saepe ex modo interdum ex 3—4 numquam e pluribus ramis extantibus. Sterigmis cacumine dentatis, conidiis cacumine dentes substringentibus.

Wenn wir nun unseren Pilz vergleichen mit *Spirotrichum purpureum*,

zeigen sich deutliche Unterschiede. Erstens wächst *Sp. purpureum* weniger langsam, die jungen Kolonien auf Bierwürze-Agar sind halbkugelig und etwa 3 mm im Durchmesser nach 14 Tagen, gegen 1—2 mm bei unserem Pilze. Die Farbe der Kolonien ist anfangs in beiden Fällen auf Bierwürze-Agar fast dieselbe, nämlich gelblich mit einem zarten, violetten Schein, doch wird sie bei unserem Pilze bald mehr gelbbraun, während *Sp. purpureum* nach einigen Wochen dunkler ist, fast gelbgrau. Ein typischer Unterschied besteht weiter darin, dass die Kolonien unseres Pilzes nach etwa 1 Monat auf Bierwürze-Agar den Nährboden anfangen aufzureissen, nach einigen Monaten hat die Kolonie ringsum Risse gezogen, sodass sie wie auf einer Insel wächst. Bei dem japanischen Pilze wurde diese Erscheinung nicht beobachtet. Schliesslich sind die Konidien bei *Sp. purpureum* mehr rundlich, bei unserem Pilze ellipsoidisch.

Die Beschreibung dieses Pilzes, den wir *Spirotrichum musae* nennen möchten, lautet folgendermaassen:

Spirotrichum musae nov. spec.

Rasen stark gefaltet, mit begrenztem Wachstum, in den Nährboden hinein wachsend und denselben schliesslich aufreissend, sammetartig, braungelb, oft mit violettem Stiche.

Konidienträger zart, 2 μ dick, nach dem Scheitel hin wirtelig verzweigt. Die Wirtel bestehen aus 3—4, oft auch aus nur 2 Sterigmen, selten gibt es mehr wie 2 Wirtel übereinander, in einer Entfernung von 30—40 μ.

Sterigmen 25—40 μ lang, 2—2,5 μ breit, gerade, mit langer, dünner Spitze, welche mehrfach hin und her gebogen ist (zackig), die Konidien an den Zacken abschnürend.

Konidien an der Spitze der Sterigmen eine Aehre bildend, indem sie an den Zacken entstehen und nach dem Loslösen zusammenkleben, ellipsoidisch, hyalin, (2,7—3,3) × (1,7—2) μ.

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 1 Monat: Kolonie 2 × 1 cm, stark wulstig, in den Agar hinein wachsend und diesen aufreissend, sammetartig, hellgelb mit volettem Stiche. Rand scharf. Schwacher Geruch. Unterseite hohl, orangerötlich, etwa 272.

Auf Röhrchen nach 1 Monat:

Auf Bierwürze- und Kirsch-Agar: Kolonien 1—1,5 mm,

zum Teil miteinander verwachsen, sammetartig, braungelblich, Farbe 222 mit 247 vermischt, mit violettem Stiche.

Auf Möhre: das Stück ganz bewachsen mit 1 mm grossen Kolonien, zum Teil eine geschlossene Decke bildend, sammetartig, braungelb, 222—247, nur teilweise mit violettem Stiche.

Auf Kartoffelstück: fast ganz bewachsen mit 1 mm grossen Kolonien, etwas spärlicher wie vorige, braungelblich, oben am Stück violett, 478 A—B.

Auf Maismehl- und Haferflocken-Agar ist das Wachstum sehr langsam, nur eine ganz kleine Kolonie von violetter Farbe, 478 B.

Hab. Isoliert von Blattflecken von Bananen in Suriname (STAHEL).

Lateinische Beschreibung:

Caespitulis limitatis numerosis rugis, saepe medium findentibus, holoserico similibus brunneis violaceo fulgore. Conidiophoris 2 μ crassis, apice ramos in coronis dispositos gerentibus. Sterigmis (25—100) × (2—2,5) μ apice dentatis. Conidiis nascentibus in dentibus, plerumque aristae instar agglutinatis, ellipsoideis hyalinis (2,7—3,3) × (1,7—2,4) μ.

Pestalotia natalensis nov. spec.

Diese *Pestalotia* wurde von Dr. MARIE LEDEBOER von einer *Acacia mollisjuna* in Natal (S. Africa) isoliert und dem C.B.S. zugeschickt. Auf künstlichen Nährböden bildet der Pilz zahlreiche Sporenlager, woraus die Sporen als grosse schwarze Tropfen hervorquellen. Diese Sporen sind fünfzellig, spindelförmig, oft mit abgerundeter Basalzelle und nicht oder nur wenig an den Querwänden eingeschnürt. Die Grösse der Sporen beträgt (25—33) × (6—9) μ; von den drei mittleren Zellen sind die beiden oberen olivbraun gefärbt, die untere nur leicht braun, während die beiden Endzellen hyalin sind. Die unterste Zelle ist mit einer stumpfen Spitze versehen oder rundlich, ein Stiel fehlt. Die oberste Zelle ist mit 3—5 langen, auseinandertretenden Zilien versehen, welche eine Länge

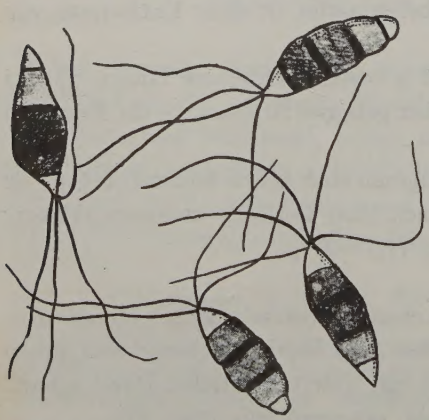

Fig. 15. *Pestalotia natalensis.* Sporen. Vergr. 750 ×.

von 40 μ erreichen; mitunter tritt an eine der Zilien ein kurzer Seitenzweig auf. Da der Pilz im Schrifttum nicht aufgefunden werden konnte und auch mit keiner der im C.B.S. vertretenen Arten übereinstimmt, wurde er als neue Art beschrieben.

Pestalotia natalensis nov. spec.

Sporenlager auf künstlichen Nährböden wie Lupinenstengel, Haferflocken-, Maismehl-, Kartoffel-Agar u.a. sehr zahlreich, mit grossen, schwarzen Sporentropfen.

Sporen spindelförmig, gerade oder etwas gekrümmt, fünfzellig, (25—33) × (6—9) μ, meist (26—27) × (7—8) μ, mit hyalinen Endzellen. Von den drei mittleren Zellen, welche zusammen im Mittel (15,3—18,7) × (8—9) μ gross sind, sind die beiden oberen olivbraun, die untere hellbraun bis leicht gefärbt. Die Basalzelle ist rundlich oder mit stumpfer Spitze versehen, ein Stiel fehlt. Die oberste Zelle trägt an der Spitze 3—5 Zilien, welche 30—40 μ lang sind und meist stark auseinander spreizen. Mitunter weist eine der Zilien einen kurzen Seitenzweig auf.

Hab. Auf der Rinde einer Akazie („Wattle") in Natal (M. Ledeboer).

Lateinische Beschreibung:

Acervulis numerosis. Conidiis fusiformibus utrinque acuminatis, rectis vel paulum curvatis, quadriseptatis, plerumque (26—27) × (7—8) μ, cellula terminali hyalina. Ex 3 mediis conidiis 2 superiora olivaceobrunnea sunt, inferius subbrunneum est, cellula basali rotunda vel obtuse acuminata apedicellata. Ciliis 3—5, 30—40 μ longis, multum divergentibus.

Zusammenfassung.

Es wurden folgende Pilze neu beschrieben:

Emericellopsis terricola, Emericellopsis terricola var. *glabra, Penicillium euglaucum, Penicillium baarnense, Penicillium novae-zeelandiae, Bisporomyces chlamydosporis, Phialophora aurantiaca, Phialophora lutea-olivacea, Graphium Cartwrightii, Oospora colorans, Spirotrichum musae* und *Pestalotia natalensis.*

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